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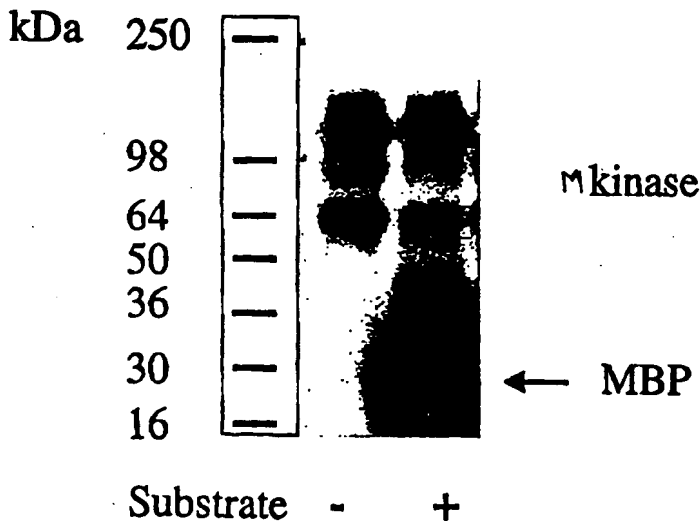
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(54) Title: TRAF4 ASSOCIATED CELL CYCLE PROTEINS, COMPOSITIONS AND METHODS OF USE



(57) Abstract: The present invention is directed to novel polypeptides, nucleic acids and related molecules which have an effect on or are related to the cell cycle. Also provided herein are vectors and host cells comprising those nucleic acid sequences, chimeric polypeptide molecules comprising the polypeptides of the present invention fused to heterologous polypeptide sequences, antibodies which bind to the polypeptides of the present invention and to methods for producing the polypeptides of the present invention. Further provided by the present invention are methods for identifying novel compositions which mediate cell cycle bioactivity, and the use of such compositions in diagnosis and treatment of disease.

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NOVEL TRAF4 ASSOCIATED CELL CYCLE PROTEINS, COMPOSITIONS AND METHODS OF USE

FIELD OF THE INVENTION

The present invention is directed to compositions involved in cell cycle regulation and methods of
10 use. More particularly, the present invention is directed to genes encoding proteins and proteins
involved in cell cycle regulation. Methods of use include use in assays screening for modulators of
the cell cycle and use as therapeutics.

BACKGROUND OF THE INVENTION

Cells cycle through various stages of growth, starting with the M phase, where mitosis and
15 cytoplasmic division (cytokinesis) occurs. The M phase is followed by the G1 phase, in which the
cells resume a high rate of biosynthesis and growth. The S phase begins with DNA synthesis, and
ends when the DNA content of the nucleus has doubled. The cell then enters G2 phase, which
ends when mitosis starts, signaled by the appearance of condensed chromosomes. Terminally
differentiated cells are arrested in the G1 phase, and no longer undergo cell division.

20 The hallmark of a malignant cell is uncontrolled proliferation. This phenotype is acquired through
the accumulation of gene mutations, the majority of which promote passage through the cell cycle.
Cancer cells ignore growth regulatory signals and remain committed to cell division. Classic
oncogenes, such as *ras*, lead to inappropriate transition from G1 to S phase of the cell cycle,
mimicking proliferative extracellular signals. Cell cycle checkpoint controls ensure faithful
25 replication and segregation of the genome. The loss of cell cycle checkpoint control results in
genomic instability, greatly accelerating the accumulation of mutations which drive malignant
transformation. Thus, modulating cell cycle checkpoint pathways and other such pathways with
therapeutic agents could exploit the differences between normal and tumor cells, both improving
the selectivity of radio- and chemotherapy, and leading to novel cancer treatments. As another
30 example, it would be useful to control entry into apoptosis.

On the other hand, it is also sometimes desirable to enhance proliferation of cells in a controlled

manner. For example, proliferation of cells is useful in wound healing and where growth of tissue is desirable. Thus, identifying modulators which promote, enhance or deter the inhibition of proliferation is desirable.

Despite the desirability of identifying cell cycle components and modulators, there is a deficit in the field of such compounds. Accordingly, it would be advantageous to provide compositions and methods useful in screening for modulators of the cell cycle. It would also be advantageous to provide novel compositions which are involved in the cell cycle.

SUMMARY OF THE INVENTION

The present invention provides cell cycle proteins and nucleic acids which encode such proteins. Also provided are methods for screening for a bioactive agent capable of modulating the cell cycle. The method comprises combining a cell cycle protein and a candidate bioactive agent and a cell or a population of cells, and determining the effect on the cell in the presence and absence of the candidate agent. Therapeutics for regulating or modulating the cell cycle are also provided.

In one aspect, a recombinant nucleic acid encoding a cell cycle protein of the present invention comprises a nucleic acid that hybridizes under high stringency conditions to a sequence complementary to that set forth in Figure 1. In a preferred embodiment, the cell cycle protein provided herein binds to Traf4.

In one embodiment, a recombinant nucleic acid is provided which comprises a nucleic acid sequence as set forth in Figure 1. In another embodiment, a recombinant nucleic acid encoding a cell cycle protein is provided which comprises a nucleic acid sequence having at least 85% sequence identity to a sequence as set forth in Figure 1. In a further embodiment, provided herein is a recombinant nucleic acid encoding an amino acid sequence as depicted in Figure 2. Preferably, the amino acid sequence has 808 amino acids as encoded from the start to the stop codon indicated in Figure 1. Preferably, fragments of the protein have at least about amino acids 14 through about 314 using the numbering starting from the first "M" shown in Figure 2.

In another aspect of the invention, expression vectors are provided. The expression vectors comprise one or more of the recombinant nucleic acids provided herein operably linked to regulatory sequences recognized by a host cell transformed with the nucleic acid. Further provided herein are host cells comprising the vectors and recombinant nucleic acids provided herein. Moreover, provided herein are processes for producing a cell cycle protein comprising culturing a host cell as described herein under conditions suitable for expression of the cell cycle protein. In one embodiment, the process includes recovering the cell cycle protein.

Also provided herein are recombinant cell cycle proteins encoded by the nucleic acids of the

present invention. In one aspect, a recombinant polypeptide is provided herein which comprises an amino acid sequence having at least 80% sequence identity with a sequence as set forth in Figure 2. In one embodiment, a recombinant cell cycle protein is provided which comprises an amino acid sequence as set forth in Figure 2.

- 5 In another aspect, the present invention provides isolated polypeptides which specifically bind to a cell cycle protein as described herein. Examples of such isolated polypeptides include antibodies. Such an antibody can be a monoclonal antibody. In one embodiment, such an antibody reduces or eliminates the biological function of said cell cycle protein.

- Further provided herein are methods for screening for a bioactive agent capable of binding to a cell
10 cycle protein. In one embodiment the method comprises combining a cell cycle protein and a candidate bioactive agent, and determining the binding of said candidate bioactive agent to said cell cycle protein.

- In another aspect, provided herein is a method for screening for a bioactive agent capable of interfering with the binding of a cell cycle protein and a Traf4 protein. In one embodiment, such a
15 method comprises combining a cell cycle protein, a candidate bioactive agent and a Traf4 protein, and determining the binding of the cell cycle protein and the Traf4 protein. If desired, the cell cycle protein and the Traf4 protein can be combined first.

- Further provided herein are methods for screening for a bioactive agent capable of modulating the activity of cell cycle protein. In one embodiment the method comprises adding a candidate
20 bioactive agent to a cell comprising a recombinant nucleic acid encoding a cell cycle protein, and determining the effect of the candidate bioactive agent on the cell. In a preferred embodiment, a library of candidate bioactive agents is added to a plurality of cells comprising a recombinant nucleic acid encoding a cell cycle protein.

- Other aspects of the invention will become apparent to the skilled artisan by the following
25 description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the nucleic acid sequence of SEQ ID NO:1, encoding a cell cycle protein MKinase, wherein the start codon and stop codon are bolded and underlined.

- Figure 2 shows the amino acid sequence of SEQ ID NO:2 which includes the sequence of a cell
30 cycle protein Mkinase. The kinase domain and nuclear localization signal are underlined.

Figure 3 shows the mRNA expression pattern of Mkinase wherein actin is used as a control.

Figure 4 shows the results of an in vitro kinase assay wherein myelin basis protein (MBP) is used as the substrate.

Figure 5 shows figures involving a full-length (FL) Mkinase and an N-terminal deleted (ND) Mkinase. Particularly, Figures 5A and 5B indicate the approximate kinase domain and nuclear localization signal (NLS) of Mkinase wherein 5A is FL and 5B is ND. 5C shows the results of an in vitro kinase activity using ND, FL or a control vector and 5D shows the results of a Western blot indicating the presence of ND and FL used in 5C.

Figure 6 shows the localization of Mkinase in Hela cells wherein 6A and 6B show staining with an anti-flag and Figures 6C and 6D show staining with DAPI.

- 10 Figure 7A shows the approximate location of the domain in Mkinase that has homology to other known kinases. Figure 7B shows a multiple sequence alignment of Mkinase from amino acids 1 through 233 against corresponding regions of other known kinases.

DETAILED DESCRIPTION OF THE INVENTION

- The present invention provides cell cycle proteins and nucleic acids which encode such proteins.
- 15 Also provided are methods for screening for a bioactive agent capable of modulating the cell cycle. The method comprises combining a cell cycle protein and a candidate bioactive agent and a cell or a population of cells, and determining the effect on the cell in the presence and absence of the candidate agent. Other screening assays including binding assays are also provided herein as described below. Therapeutics for regulating or modulating the cell cycle are also provided and
- 20 described herein. Diagnostics, as further described below, are also provided herein.

- A cell cycle protein of the present invention may be identified in several ways. "Protein" in this sense includes proteins, polypeptides, and peptides. The cell cycle proteins of the invention fall into two general classes: proteins that are completely novel, i.e. are not part of a public database as of the time of discovery, although they may have homology to either known proteins or peptides
- 25 encoded by expressed sequence tags (ESTs). Alternatively, the cell cycle proteins are known proteins, but that were not known to be involved in the cell cycle; i.e. they are identified herein as having a novel biological function. Accordingly, a cell cycle protein may be initially identified by its association with a protein known to be involved in the cell cycle. Wherein the cell cycle proteins and nucleic acids are novel, compositions and methods of use are provided herein. In the case
- 30 that the cell cycle proteins and nucleic acids were known but not known to be involved in cell cycle activity as described herein, methods of use, i.e. functional screens, are provided.

In one embodiment provided herein, a cell cycle protein as defined herein has one or more of the following characteristics: binding to Traf4; homology to protein kinases, preferably protein kinases

associated with microtubules and/or cell cycling; and cell cycle protein activity as described herein.

The homology to such kinases can be found as described below. In one embodiment, homology is found using the following database and parameters. Database: Non-redundant GenBank CDS translations+PDB+SwissProt+SPupdate+PIR; Lambda of 0.316, K of 0.133 and H of 0; Gapped

- 5 Lambda of 0.27, K of 0.047, and H of 4.94e-324; Matrix is BLOSUM62; Gap Penalties: Existence: 11, Extension: 1.

In one embodiment, the cell cycle protein is termed Mkinase herein. The characteristics described below can apply to any of the cell cycle proteins provided herein, however, Mkinase is used for illustrative purposes. Mkinase has similarity to proteins belonging to a family of kinases and has a

- 10 kinase domain in its N-terminal. Preferably, Radh binds to members of the tumor necrosis factor receptor associated factor (TRAF) family, preferably Traf4. Traf4 expression may be observed during embryogenesis, mostly in the central nervous system and peripheral nervous system, and remain expressed through adulthood, primarily in the hippocampus and olfactory bulb. Masson, et al., Mech. Dev., 71(1-2):187-91 (1998). Studies have also reported that Traf4 expression exists in
- 15 normal epithelial stem cells and expression of such ceases upon differentiation and malignant transformation of cells. Moreover, Traf4 expression can also be found in breast carcinomas. Krajewska, et al., Am J. Pathol., 152(6):1549-61 (1998), Tomasetto, et al., Am J Pathol., 153(6):2007-8 (1998).

- Furthermore, regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-
- 20 oligomerization is described in, e.g., Pullen, et al., Biochemistry, 37(34):11836-45 (1998); Pullen, et al., J Biol Chem., 274(20):14246-54 (1999); Ishida, et al., PNAS USA, 93(18):9437-42 (1996); Kashiwada, et al., J Exp Med, 187(2):237-44(1998). Additionally, cell cycle and apoptosis-related proteins, kinases, and carcinomas are described in Muzio, et al., J Dent Res., 78(7):1345-53 (1999); Jimenez, et al., Nature, 400(6739):81-83 (1999); and Hsieh, Int J Oncol., 15(2):245-252
- 25 (1999).

In a preferred embodiment, Mkinase has a kinase domain in its N-terminal. Preferably, Mkinase shares homology with map kinase families and CDK families. Most preferably, Mkinase shares homology with protein kinases associated with microtubules and cell cycling. The novel cell cycle proteins provided herein share greater homology with the sequences in the figures than do the

30 kinases described below or other known proteins. A study reports on MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. Drewes, et al., Cell, 89(2):297-308 (1997). Moreover, studies report on lack of elevated MAP (Erk) activity in pancreatic carcinomas, blockage of MAP kinase pathways suppress colon tumors and MAP involvement in apoptosis and cell activation. Yip-Schneider, Int J Oncol.,

35 15(2):271-279 (1999); Sebolt-Leopold, Nat Med., 5(7):810-6 (1999); and Birkenkamp, et al., Leukemia, 13(7):1037-45 (1999). Regarding MAP, also see, Nguyen and Shiozaki, Genes Dev., 13(13):1653-1663 (1999). Moreover, regarding cdc2-related kinases, see, Kinnaird, et al., Mol

In one embodiment, cell cycle nucleic acids or cell cycle proteins are initially identified by substantial nucleic acid and/or amino acid sequence identity or similarity to the sequence(s) provided herein. In a preferred embodiment, cell cycle nucleic acids or cell cycle proteins have
5 sequence identity or similarity to the sequences provided herein as described below and one or more of the cell cycle protein bioactivities as further described below. Such sequence identity or similarity can be based upon the overall nucleic acid or amino acid sequence.

In a preferred embodiment, a protein is a "cell cycle protein" as defined herein if the overall sequence identity of the amino acid sequence of Figure 2 is preferably greater than about 75%,
10 more preferably greater than about 80%, even more preferably greater than about 85% and most preferably greater than 90%. In some embodiments the sequence identity will be as high as about 93 to 95 or 98%.

In another preferred embodiment, a cell cycle protein has an overall sequence similarity with the amino acid sequence of Figure 2 of greater than about 80%, more preferably greater than about
15 85%, even more preferably greater than about 90% and most preferably greater than 93%. In some embodiments the sequence identity will be as high as about 95 to 98 or 99%.

As is known in the art, a number of different programs can be used to identify whether a protein (or nucleic acid as discussed below) has sequence identity or similarity to a known sequence. Sequence identity and/or similarity is determined using standard techniques known in the art,
20 including, but not limited to, the local sequence identity algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, PNAS USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and
TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science
25 Drive, Madison, WI), the Best Fit sequence program described by Devereux *et al.*, Nucl. Acid Res. 12:387-395 (1984), preferably using the default settings, or by inspection. Preferably, percent identity is calculated by FastDB based upon the following parameters: mismatch penalty of 1; gap
penalty of 1; gap size penalty of 0.33; and joining penalty of 30, "Current Methods in Sequence
Comparison and Analysis," Macromolecule Sequencing and Synthesis, Selected Methods and
30 Applications, pp 127-149 (1988), Alan R. Liss, Inc.

An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol. 35:351-360 (1987); the method
35 is similar to that described by Higgins & Sharp CABIOS 5:151-153 (1989). Useful PILEUP

parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

Another example of a useful algorithm is the BLAST algorithm, described in Altschul et al., J. Mol. Biol. 215, 403-410, (1990) and Karlin et al., PNAS USA 90:5873-5787 (1993). A particularly useful
5 BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al., Methods in Enzymology, 266: 460-480 (1996); [http://blast.wustl.edu/blast/ README.html](http://blast.wustl.edu/blast/README.html). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program
10 itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

An additional useful algorithm is gapped BLAST as reported by Altschul et al. Nucleic Acids Res. 25:3389-3402. Gapped BLAST uses BLOSUM-62 substitution scores; threshold *T* parameter set
15 to 9; the two-hit method to trigger ungapped extensions; charges gap lengths of *k* a cost of 10+*k*; *X_u* set to 16, and *X_o* set to 40 for database search stage and to 67 for the output stage of the algorithms. Gapped alignments are triggered by a score corresponding to ~22 bits.

A % amino acid sequence identity value is determined by the number of matching identical
20 residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

In a similar manner, "percent (%) nucleic acid sequence identity" with respect to the coding sequence of the polypeptides identified herein is defined as the percentage of nucleotide residues
25 in a candidate sequence that are identical with the nucleotide residues in the coding sequence of the cell cycle protein. A preferred method utilizes the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the protein encoded by the
30 sequences in the Figures, it is understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acids in relation to the total number of amino acids. Thus, for example, sequence identity of sequences shorter than that shown in the Figure, as discussed below, will be determined using the number of amino acids in the shorter sequence, in one embodiment. In percent identity calculations relative weight is not
35 assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, etc.

In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0", which obviates the need for a weighted scale or parameters as described below for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total
5 number of residues of the "shorter" sequence in the aligned region and multiplying by 100. The "longer" sequence is the one having the most actual residues in the aligned region.

As will be appreciated by those skilled in the art, the sequences of the present invention may contain sequencing errors. That is, there may be incorrect nucleosides, frameshifts, unknown nucleosides, or other types of sequencing errors in any of the sequences; however, the correct
10 sequences will fall within the homology and stringency definitions herein.

Cell cycle proteins of the present invention may be shorter or longer than the amino acid sequence encoded by the nucleic acid shown in the Figure. Thus, in a preferred embodiment, included within the definition of cell cycle proteins are portions or fragments of the amino acid sequence encoded by the nucleic acid sequence provided herein. In one embodiment herein, fragments of cell cycle
15 proteins are considered cell cycle proteins if a) they share at least one antigenic epitope; b) have at least the indicated sequence identity; c) and preferably have cell cycle biological activity as further defined herein. In some cases, where the sequence is used diagnostically, that is, when the presence or absence of cell cycle protein nucleic acid is determined, only the indicated sequence identity is required. The nucleic acids of the present invention may also be shorter or longer than
20 the sequence in the Figure. The nucleic acid fragments include any portion of the nucleic acids provided herein which have a sequence not exactly previously identified; fragments having sequences with the indicated sequence identity to that portion not previously identified are provided in an embodiment herein.

In addition, as is more fully outlined below, cell cycle proteins can be made that are longer than
25 those depicted in the Figure; for example, by the addition of epitope or purification tags, the addition of other fusion sequences, or the elucidation of additional coding and non-coding sequences. As described below, the fusion of a cell cycle peptide to a fluorescent peptide, such as Green Fluorescent Peptide (GFP), is particularly preferred.

Cell cycle proteins may also be identified as encoded by cell cycle nucleic acids which hybridize to
30 the sequence depicted in the Figure, or the complement thereof, as outlined herein. Hybridization conditions are further described below.

In a preferred embodiment, when a cell cycle protein is to be used to generate antibodies, a cell cycle protein must share at least one epitope or determinant with the full length protein. By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an
35 antibody. Thus, in most instances, antibodies made to a smaller cell cycle protein will be able to

bind to the full length protein. In a preferred embodiment, the epitope is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity. The term "antibody" includes antibody fragments, as are known in the art, including Fab Fab₂, single chain antibodies (Fv for example), chimeric antibodies, etc., either produced by the modification of whole antibodies or
5 those synthesized de novo using recombinant DNA technologies.

In a preferred embodiment, the antibodies to a cell cycle protein are capable of reducing or eliminating the biological function of the cell cycle proteins described herein, as is described below. That is, the addition of anti-cell cycle protein antibodies (either polyclonal or preferably monoclonal) to cell cycle proteins (or cells containing cell cycle proteins) may reduce or eliminate the cell cycle
10 activity. Generally, at least a 25% decrease in activity is preferred, with at least about 50% being particularly preferred and about a 95-100% decrease being especially preferred.

The cell cycle antibodies of the invention specifically bind to cell cycle proteins. In a preferred embodiment, the antibodies specifically bind to cell cycle proteins. By "specifically bind" herein is meant that the antibodies bind to the protein with a binding constant in the range of at least 10^{-4} -
15 10^{-6} M⁻¹, with a preferred range being 10^{-7} - 10^{-9} M⁻¹. Antibodies are further described below.

In the case of the nucleic acid, the overall sequence identity of the nucleic acid sequence is commensurate with amino acid sequence identity but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the nucleic acid sequence identity may be either lower or higher than that of the protein sequence. Thus the sequence
20 identity of the nucleic acid sequence as compared to the nucleic acid sequence of the Figure is preferably greater than 75%, more preferably greater than about 80%, particularly greater than about 85% and most preferably greater than 90%. In some embodiments the sequence identity will be as high as about 93 to 95 or 98%.

In a preferred embodiment, a cell cycle nucleic acid encodes a cell cycle protein. As will be
25 appreciated by those in the art, due to the degeneracy of the genetic code, an extremely large number of nucleic acids may be made, all of which encode the cell cycle proteins of the present invention. Thus, having identified a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids, by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the cell cycle protein.

30 In one embodiment, the nucleic acid is determined through hybridization studies. Thus, for example, nucleic acids which hybridize under high stringency to the nucleic acid sequence shown in the Figure, or its complement is considered a cell cycle nucleic acid. High stringency conditions are known in the art; see for example Maniatis et al., Molecular Cloning: A Laboratory Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, et al., both of which are
35 hereby incorporated by reference. Stringent conditions are sequence-dependent and will be

- different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally,
- 5 stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in
- 10 which the salt concentration is less than about 1.0 sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.
- 15 In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Maniatis and Ausubel, supra, and Tijssen, supra.

- The cell cycle proteins and nucleic acids of the present invention are preferably recombinant. As used herein and further defined below, "nucleic acid" may refer to either DNA or RNA, or molecules
- 20 which contain both deoxy- and ribonucleotides. The nucleic acids include genomic DNA, cDNA and oligonucleotides including sense and anti-sense nucleic acids. Such nucleic acids may also contain modifications in the ribose-phosphate backbone to increase stability and half life of such molecules in physiological environments.

- The nucleic acid may be double stranded, single stranded, or contain portions of both double
- 25 stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand ("Watson") also defines the sequence of the other strand ("Crick"); thus the sequences depicted in the Figures also include the complement of the sequence. By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid by endonucleases, in a form not normally found in nature. Thus an
- 30 isolated cell cycle nucleic acid, in a linear form, or an expression vector formed *in vitro* by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e. using the *in vivo* cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced
- 35 recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of a cell cycle protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of an inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Alternatively, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid substitutions, insertions and deletions, as discussed below.

In one embodiment, the present invention provides cell cycle protein variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding a cell cycle protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant cell cycle protein fragments having up to about 100-150 residues may be prepared by *in vitro* synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the cell cycle protein amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will be more fully outlined below.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed cell cycle variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants is done using assays of cell cycle protein activities.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated.

Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the cell cycle protein are desired, substitutions are generally made in accordance with the following chart:

Chart I

<u>Original Residue</u>	<u>Exemplary Substitutions</u>
10 Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
15 Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
20 Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
25 Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or

by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the cell cycle proteins as needed. Alternatively, the variant may be designed such that the biological activity of the cell cycle protein is altered. For example, glycosylation sites may be altered or removed.

- Covalent modifications of cell cycle polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of a cell cycle polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of a cell cycle polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking cell cycle to a water-insoluble support matrix or surface for use in the method for purifying anti-cell cycle antibodies or screening assays, as is more fully described below. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azido-salicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propionimidate.
- Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

- Another type of covalent modification of the cell cycle polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence cell cycle polypeptide, and/or adding one or more glycosylation sites that are not present in the native sequence cell cycle polypeptide.

- Addition of glycosylation sites to cell cycle polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence cell cycle polypeptide (for O-linked glycosylation sites). The cell cycle amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the cell cycle polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the cell cycle polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

- 5 Removal of carbohydrate moieties present on the cell cycle polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties
10 on polypeptides can be achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

- Another type of covalent modification of cell cycle comprises linking the cell cycle polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144;
15 4,670,417; 4,791,192 or 4,179,337.

- Cell cycle polypeptides of the present invention may also be modified in a way to form chimeric molecules comprising a cell cycle polypeptide fused to another, heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of a cell cycle polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can
20 selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the cell cycle polypeptide. The presence of such epitope-tagged forms of a cell cycle polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the cell cycle polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In an alternative embodiment, the
25 chimeric molecule may comprise a fusion of a cell cycle polypeptide with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule as discussed further below.

- Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag
30 polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope
35 peptide [Martin et al., Science, 255:192-194 (1992)]; tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al.,

In an embodiment herein, cell cycle proteins of the cell cycle family and cell cycle proteins from other organisms are cloned and expressed as outlined below. Thus, probe or degenerate polymerase chain reaction (PCR) primer sequences may be used to find other related cell cycle
5 proteins from humans or other organisms. As will be appreciated by those in the art, particularly useful probe and/or PCR primer sequences include the unique areas of the cell cycle nucleic acid sequence. As is generally known in the art, preferred PCR primers are from about 15 to about 35 nucleotides in length, with from about 20 to about 30 being preferred, and may contain inosine as needed. The conditions for the PCR reaction are well known in the art. It is therefore also
10 understood that provided along with the sequences in the sequences listed herein are portions of those sequences, wherein unique portions of 15 nucleotides or more are particularly preferred. The skilled artisan can routinely synthesize or cut a nucleotide sequence to the desired length.

Once isolated from its natural source, e.g., contained within a plasmid or other vector or excised therefrom as a linear nucleic acid segment, the recombinant cell cycle nucleic acid can be further-
15 used as a probe to identify and isolate other cell cycle nucleic acids. It can also be used as a "precursor" nucleic acid to make modified or variant cell cycle nucleic acids and proteins.

Using the nucleic acids of the present invention which encode a cell cycle protein, a variety of expression vectors are made. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome. Generally, these
20 expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the cell cycle protein. The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are
25 known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the
30 polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. As another example, operably linked refers to DNA sequences linked so as to be contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by
35 ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. The transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used to express

the cell cycle protein; for example, transcriptional and translational regulatory nucleic acid sequences from *Bacillus* are preferably used to express the cell cycle protein in *Bacillus*.

Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells.

- 5 In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In a preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences.

- 10 Promoter sequences encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention.

- In addition, the expression vector may comprise additional elements. For example, the expression
15 vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a procaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences which flank the expression construct. The integrating vector may be directed to a
20 specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art.

In addition, in a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used.

- 25 A preferred expression vector system is a retroviral vector system such as is generally described in PCT/US97/01019 and PCT/US97/01048, both of which are hereby expressly incorporated by reference.

- Cell cycle proteins of the present invention are produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding a cell cycle protein, under the appropriate
30 conditions to induce or cause expression of the cell cycle protein. The conditions appropriate for cell cycle protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation. For example, the use of constitutive promoters in the expression vector will require optimizing the growth and proliferation of the host cell, while the use of an inducible promoter requires the

appropriate growth conditions for induction. In addition, in some embodiments, the timing of the harvest is important. For example, the baculoviral systems used in insect cell expression are lytic viruses, and thus harvest time selection can be crucial for product yield.

Appropriate host cells include yeast, bacteria, archebacteria, fungi, and insect and animal cells, including mammalian cells. Of particular interest are *Drosophila melanogaster* cells, *Saccharomyces cerevisiae* and other yeasts, *E. coli*, *Bacillus subtilis*, SF9 cells, C129 cells, 293 cells, Neurospora, BHK, CHO, COS, and HeLa cells, fibroblasts, Schwannoma cell lines, immortalized mammalian myeloid and lymphoid cell lines and tumor cell lines.

In a preferred embodiment, the cell cycle proteins are expressed in mammalian cells. Mammalian expression systems are also known in the art, and include retroviral systems. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence for cell cycle protein into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, using a located 25-30 base pairs upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter.

Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenylation signals include those derived from SV40.

The methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, is well known in the art, and will vary with the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, viral infection, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

In a preferred embodiment, cell cycle proteins are expressed in bacterial systems. Bacterial expression systems are well known in the art.

A suitable bacterial promoter is any nucleic acid sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of the coding sequence of cell cycle protein into mRNA. A bacterial promoter has a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region typically includes
5 an RNA polymerase binding site and a transcription initiation site. Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose and maltose, and sequences derived from biosynthetic enzymes such as tryptophan. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters
10 are also useful; for example, the *tac* promoter is a hybrid of the *trp* and *lac* promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription.

In addition to a functioning promoter sequence, an efficient ribosome binding site is desirable. In *E.*
15 *coli*, the ribosome binding site is called the Shine-Delgarno (SD) sequence and includes an initiation codon and a sequence 3-9 nucleotides in length located 3 - 11 nucleotides upstream of the initiation codon.

The expression vector may also include a signal peptide sequence that provides for secretion of the cell cycle protein in bacteria. The signal sequence typically encodes a signal peptide
20 comprised of hydrophobic amino acids which direct the secretion of the protein from the cell, as is well known in the art. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria).

The bacterial expression vector may also include a selectable marker gene to allow for the
25 selection of bacterial strains that have been transformed. Suitable selection genes include genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways.

These components are assembled into expression vectors. Expression vectors for bacteria are
30 well known in the art, and include vectors for *Bacillus subtilis*, *E. coli*, *Streptococcus cremoris*, and *Streptococcus lividans*, among others.

The bacterial expression vectors are transformed into bacterial host cells using techniques well known in the art, such as calcium chloride treatment, electroporation, and others.

In one embodiment, cell cycle proteins are produced in insect cells. Expression vectors for the
35 transformation of insect cells, and in particular, baculovirus-based expression vectors, are well

known in the art.

In a preferred embodiment, cell cycle protein is produced in yeast cells. Yeast expression systems are well known in the art, and include expression vectors for *Saccharomyces cerevisiae*, *Candida albicans* and *C. maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis* and *K. lactis*, *Pichia guillermondii* and *P. pastoris*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*. Preferred promoter sequences for expression in yeast include the inducible GAL1,10 promoter, the promoters from alcohol dehydrogenase, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase, hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, pyruvate kinase, and the acid phosphatase gene. Yeast selectable markers include ADE2, HIS4, LEU2, TRP1, and ALG7, which confers resistance to tunicamycin; the neomycin phosphotransferase gene, which confers resistance to G418; and the CUP1 gene, which allows yeast to grow in the presence of copper ions.

The cell cycle protein may also be made as a fusion protein, using techniques well known in the art. Thus, for example, for the creation of monoclonal antibodies, if the desired epitope is small, the cell cycle protein may be fused to a carrier protein to form an immunogen. Alternatively, the cell cycle protein may be made as a fusion protein to increase expression, or for other reasons. For example, when the cell cycle protein is a cell cycle peptide, the nucleic acid encoding the peptide may be linked to other nucleic acid for expression purposes. Similarly, cell cycle proteins of the invention can be linked to protein labels, such as green fluorescent protein (GFP), red fluorescent protein (RFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), etc.

In one embodiment, the cell cycle nucleic acids, proteins and antibodies of the invention are labeled. By "labeled" herein is meant that a compound has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the compound at any position.

In a preferred embodiment, the cell cycle protein is purified or isolated after expression. Cell cycle proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the cell cycle protein may be purified using a standard anti-cell cycle antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable purification techniques, see Scopes, R., Protein Purification, Springer-Verlag, NY (1982). The degree of purification necessary will vary depending on the use of the cell cycle protein. In some instances no purification will be necessary.

Once expressed and purified if necessary, the cell cycle proteins and nucleic acids are useful in a number of applications.

The nucleotide sequences (or their complement) encoding cell cycle proteins have various applications in the art of molecular biology, including uses as hybridization probes, in chromosome
5 and gene mapping and in the generation of anti-sense RNA and DNA. Cell cycle protein nucleic acid will also be useful for the preparation of cell cycle proteins by the recombinant techniques described herein.

The full-length native sequence cell cycle protein gene, or portions thereof, may be used as hybridization probes for a cDNA library to isolate other genes (for instance, those encoding
10 naturally-occurring variants of cell cycle protein or cell cycle protein from other species) which have a desired sequence identity to the cell cycle protein coding sequence. Optionally, the length of the probes will be about 20 to about 50 bases. The hybridization probes may be derived from the nucleotide sequences herein or from genomic sequences including promoters, enhancer elements and introns of native sequences as provided herein. By way of example, a screening method will
15 comprise isolating the coding region of the cell cycle protein gene using the known DNA sequence to synthesize a selected probe of about 40 bases. Hybridization probes may be labeled by a variety of labels, including radionucleotides such as ³²P or ³⁵S, or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems. Labeled probes having a sequence complementary to that of the cell cycle protein gene of the present invention can be used
20 to screen libraries of human cDNA, genomic DNA or mRNA to determine which members of such libraries the probe hybridizes.

Nucleotide sequences encoding a cell cycle protein can also be used to construct hybridization probes for mapping the gene which encodes that cell cycle protein and for the genetic analysis of individuals with genetic disorders. The nucleotide sequences provided herein may be mapped to a
25 chromosome and specific regions of a chromosome using known techniques, such as *in situ* hybridization, linkage analysis against known chromosomal markers, and hybridization screening with libraries.

Nucleic acids which encode cell cycle protein or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and
30 screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding a cell cycle protein can be used to clone genomic DNA encoding a cell cycle
35 protein in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express the desired DNA. Methods for generating

transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for the cell cycle protein transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding a cell cycle protein

5 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of the desired nucleic acid. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with its overexpression. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to

10 untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition.

Alternatively, non-human homologues of the cell cycle protein can be used to construct a cell cycle protein "knock out" animal which has a defective or altered gene encoding a cell cycle protein as a

15 result of homologous recombination between the endogenous gene encoding a cell cycle protein and altered genomic DNA encoding a cell cycle protein introduced into an embryonic cell of the animal. For example, cDNA encoding a cell cycle protein can be used to clone genomic DNA encoding a cell cycle protein in accordance with established techniques. A portion of the genomic DNA encoding a cell cycle protein can be deleted or replaced with another gene, such as a gene

20 encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, *Cell*, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are

25 selected [see e.g., Li et al., *Cell*, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out"

30 animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the cell cycle protein.

35 It is understood that the models described herein can be varied. For example, "knock-in" models can be formed, or the models can be cell-based rather than animal models.

Nucleic acid encoding the cell cycle polypeptides, antagonists or agonists may also be used in gene therapy. In gene therapy applications, genes are introduced into cells in order to achieve *in*

- vivo synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense
- 5 RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes *in vivo*. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik *et al.*, Proc. Natl. Acad. Sci. USA **83**, 4143-4146 [1986]). The oligonucleotides can be modified to enhance their uptake, e.g. by substituting their negatively
- 10 charged phosphodiester groups by uncharged groups.

- There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion,
- 15 DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau *et al.*, Trends in Biotechnology **11**, 205-210 [1993]). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a
- 20 ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is
- 25 described, for example, by Wu *et al.*, J. Biol. Chem. **262**, 4429-4432 (1987); and Wagner *et al.*, Proc. Natl. Acad. Sci. USA **87**, 3410-3414 (1990). For review of gene marking and gene therapy protocols see Anderson *et al.*, Science **256**, 808-813 (1992).

- In a preferred embodiment, the cell cycle proteins, nucleic acids, variants, modified proteins, cells and/or transgenics containing the said nucleic acids or proteins are used in screening assays.
- 30 Identification of the cell cycle protein provided herein permits the design of drug screening assays for compounds that bind or interfere with the binding to the cell cycle protein and for compounds which modulate cell cycle activity.

- The assays described herein preferably utilize the human cell cycle protein, although other mammalian proteins may also be used, including rodents (mice, rats, hamsters, guinea pigs, etc.),
- 35 farm animals (cows, sheep, pigs, horses, etc.) and primates. These latter embodiments may be preferred in the development of animal models of human disease. In some embodiments, as outlined herein, variant or derivative cell cycle proteins may be used, including deletion cell cycle

proteins as outlined above.

In a preferred embodiment, the methods comprise combining a cell cycle protein and a candidate bioactive agent, and determining the binding of the candidate agent to the cell cycle protein. In other embodiments, further discussed below, binding interference or bioactivity is determined.

- 5 The term "candidate bioactive agent" or "exogeneous compound" as used herein describes any molecule, e.g., protein, small organic molecule, carbohydrates (including polysaccharides), polynucleotide, lipids, etc. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration or below the level
10 of detection. In addition, positive controls, i.e. the use of agents known to alter cell cycling, may be used. For example, p21 is a molecule known to arrest cells in the G1 cell phase, by binding G1 cyclin-CDK complexes.

- Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and
15 less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups.
20 Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

- Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed
25 synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to
30 directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

- In a preferred embodiment, a library of different candidate bioactive agents are used. Preferably, the library should provide a sufficiently structurally diverse population of randomized agents to effect a probabilistically sufficient range of diversity to allow binding to a particular target.
35 Accordingly, an interaction library should be large enough so that at least one of its members will

- have a structure that gives it affinity for the target. Although it is difficult to gauge the required absolute size of an interaction library, nature provides a hint with the immune response: a diversity of 10^7 - 10^8 different antibodies provides at least one combination with sufficient affinity to interact with most potential antigens faced by an organism. Published *in vitro* selection techniques have
- 5 also shown that a library size of 10^7 to 10^8 is sufficient to find structures with affinity for the target. A library of all combinations of a peptide 7 to 20 amino acids in length, such as generally proposed herein, has the potential to code for 20^7 (10^9) to 20^{20} . Thus, with libraries of 10^7 to 10^8 different molecules the present methods allow a "working" subset of a theoretically complete interaction library for 7 amino acids, and a subset of shapes for the 20^{20} library. Thus, in a preferred
- 10 embodiment, at least 10^6 , preferably at least 10^7 , more preferably at least 10^8 and most preferably at least 10^9 different sequences are simultaneously analyzed in the subject methods. Preferred methods maximize library size and diversity.

- In a preferred embodiment, the candidate bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides,
- 15 oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homophenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline.
- 20 The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard *in vivo* degradations. Chemical blocking groups or other chemical substituents may also be added.

- In a preferred embodiment, the candidate bioactive agents are naturally occurring proteins or
- 25 fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eukaryotic proteins may be made for screening in the systems described herein. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

- 30 In a preferred embodiment, the candidate bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random
- 35 nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or

nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous agents.

In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment, the candidate bioactive agents are nucleic acids. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage, *et al.*, *Tetrahedron*, 49(10):1925 (1993) and references therein; Letsinger, *J. Org. Chem.*, 35:3800 (1970); Sprinzl, *et al.*, *Eur. J. Biochem.*, 81:579 (1977); Letsinger, *et al.*, *Nucl. Acids Res.*, 14:3487 (1986); Sawai, *et al.*, *Chem. Lett.*, 805 (1984), Letsinger, *et al.*, *J. Am. Chem. Soc.*, 110:4470 (1988); and Pauwels, *et al.*, *Chemica Scripta*, 26:141 (1986)), phosphorothioate (Mag, *et al.*, *Nucleic Acids Res.*, 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu, *et al.*, *J. Am. Chem. Soc.*, 111:2321 (1989)), O-methylphosphoroamidite linkages (see Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, *J. Am. Chem. Soc.*, 114:1895 (1992); Meier, *et al.*, *Chem. Int. Ed. Engl.*, 31:1008 (1992); Nielsen, *Nature*, 365:566 (1993); Carlsson, *et al.*, *Nature*, 380:207 (1996), all of which are incorporated by reference)). Other analog nucleic acids include those with positive backbones (Denpcy, *et al.*, *Proc. Natl. Acad. Sci. USA*, 92:6097 (1995)); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowshi, *et al.*, *Angew. Chem. Intl. Ed. English*, 30:423 (1991); Letsinger, *et al.*, *J. Am. Chem. Soc.*, 110:4470 (1988); Letsinger, *et al.*, *Nucleoside & Nucleotide*, 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, *et al.*, *Bioorganic & Medicinal Chem. Lett.*, 4:395 (1994); Jeffs, *et al.*, *J. Biomolecular NMR*, 34:17 (1994); *Tetrahedron Lett.*, 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins, *et al.*, *Chem. Soc. Rev.*, (1995) pp. 169-176). Several nucleic acid analogs are described in Rawls, C & E News, June 2, 1997, page 35. All of these references are hereby expressly incorporated by reference. These modifications of the

ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and
5 analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc.

- 10 As described above generally for proteins, nucleic acid candidate bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eukaryotic genomes may be used as is outlined above for proteins.

- In a preferred embodiment, the candidate bioactive agents are organic chemical moieties, a wide
15 variety of which are available in the literature.

- In a preferred embodiment, the candidate bioactive agents are linked to a fusion partner. By "fusion partner" or "functional group" herein is meant a sequence that is associated with the candidate bioactive agent, that confers upon all members of the library in that class a common function or ability. Fusion partners can be heterologous (i.e. not native to the host cell), or
20 synthetic (not native to any cell). Suitable fusion partners include, but are not limited to: a) presentation structures, which provide the candidate bioactive agents in a conformationally restricted or stable form; b) targeting sequences, which allow the localization of the candidate bioactive agent into a subcellular or extracellular compartment; c) rescue sequences which allow the purification or isolation of either the candidate bioactive agents or the nucleic acids encoding
25 them; d) stability sequences, which confer stability or protection from degradation to the candidate bioactive agent or the nucleic acid encoding it, for example resistance to proteolytic degradation; e) dimerization sequences, to allow for peptide dimerization; or f) any combination of a), b), c), d), and e), as well as linker sequences as needed.

- In one embodiment of the methods described herein, portions of cell cycle proteins are utilized; in a
30 preferred embodiment, portions having cell cycle activity are used. Cell cycle activity is described further below and includes binding activity to a TRAF protein or cell cycle protein modulators as further described below. In addition, the assays described herein may utilize either isolated cell cycle proteins or cells comprising the cell cycle proteins.

- Generally, in a preferred embodiment of the methods herein, for example for binding assays, the
35 cell cycle protein or the candidate agent is non-diffusibly bound to an insoluble support having

- isolated sample receiving areas (e.g. a microtiter plate, an array, etc.). The insoluble supports may be made of any composition to which the compositions can be bound, is readily separated from soluble material, and is otherwise compatible with the overall method of screening. The surface of such supports may be solid or porous and of any convenient shape. Examples of
- 5 suitable insoluble supports include microtiter plates, arrays, membranes and beads. These are typically made of glass, plastic (e.g., polystyrene), polysaccharides, nylon or nitrocellulose, teflon™, etc. Microtiter plates and arrays are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. In some cases magnetic beads and the like are included. The particular manner of binding of the
- 10 composition is not crucial so long as it is compatible with the reagents and overall methods of the invention, maintains the activity of the composition and is nondiffusible. Preferred methods of binding include the use of antibodies (which do not sterically block either the ligand binding site or activation sequence when the protein is bound to the support), direct binding to "sticky" or ionic supports, chemical crosslinking, the synthesis of the protein or agent on the surface, etc. In some
- 15 embodiments, Traf4 can be used. Following binding of the protein or agent, excess unbound material is removed by washing. The sample receiving areas may then be blocked through incubation with bovine serum albumin (BSA), casein or other innocuous protein or other moiety. Also included in this invention are screening assays wherein solid supports are not used; examples of such are described below.
- 20 In a preferred embodiment, the cell cycle protein is bound to the support, and a candidate bioactive agent is added to the assay. Alternatively, the candidate agent is bound to the support and the cell cycle protein is added. Novel binding agents include specific antibodies, non-natural binding agents identified in screens of chemical libraries, peptide analogs, etc. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may
- 25 be used for this purpose, including labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.) and the like.

- The determination of the binding of the candidate bioactive agent to the cell cycle protein may be done in a number of ways. In a preferred embodiment, the candidate bioactive agent is labelled,
- 30 and binding determined directly. For example, this may be done by attaching all or a portion of the cell cycle protein to a solid support, adding a labelled candidate agent (for example a fluorescent label), washing off excess reagent, and determining whether the label is present on the solid support. Various blocking and washing steps may be utilized as is known in the art.

- By "labeled" herein is meant that the compound is either directly or indirectly labeled with a label
- 35 which provides a detectable signal, e.g. radioisotope, fluorescers, enzyme, antibodies, particles such as magnetic particles, chemilumescers, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the

specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal.

5 In some embodiments, only one of the components is labeled. For example, the proteins (or proteinaceous candidate agents) may be labeled at tyrosine positions using ^{125}I , or with fluorophores. Alternatively, more than one component may be labeled with different labels; using ^{125}I for the proteins, for example, and a fluorophor for the candidate agents.

10 In a preferred embodiment, the binding of the candidate bioactive agent is determined through the use of competitive binding assays. In this embodiment, the competitor is a binding moiety known to bind to the target molecule (i.e. cell cycle protein), such as an antibody, peptide, binding partner, ligand, etc. In a preferred embodiment, the competitor is Traf4. Under certain circumstances, there may be competitive binding as between the bioactive agent and the binding moiety, with the binding moiety displacing the bioactive agent. This assay can be used to determine candidate agents which interfere with binding between cell cycle proteins and Traf4. "Interference of binding" 15 as used herein means that native binding of the cell cycle protein differs in the presence of the candidate agent. The binding can be eliminated or can be with a reduced affinity. Therefore, in one embodiment, interference is caused by, for example, a conformation change, rather than direct competition for the native binding site.

20 In one embodiment, the candidate bioactive agent is labeled. Either the candidate bioactive agent, or the competitor, or both, is added first to the protein for a time sufficient to allow binding, if present. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high throughput screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The second component is then 25 added, and the presence or absence of the labeled component is followed, to indicate binding.

In a preferred embodiment, the competitor is added first, followed by the candidate bioactive agent. Displacement of the competitor is an indication that the candidate bioactive agent is binding to the cell cycle protein and thus is capable of binding to, and potentially modulating, the activity of the cell cycle protein. In this embodiment, either component can be labeled. Thus, for example, if the 30 competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the candidate bioactive agent is labeled, the presence of the label on the support indicates displacement.

In an alternative embodiment, the candidate bioactive agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate that 35 the bioactive agent is bound to the cell cycle protein with a higher affinity. Thus, if the candidate

bioactive agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate that the candidate agent is capable of binding to the cell cycle protein.

In a preferred embodiment, the methods comprise differential screening to identify bioactive agents
5 that are capable of modulating the activity of the cell cycle proteins. Such assays can be done with the cell cycle protein or cells comprising said cell cycle protein. In one embodiment, the methods comprise combining an cell cycle protein and a competitor in a first sample. A second sample comprises a candidate bioactive agent, an cell cycle protein and a competitor. The binding of the competitor is determined for both samples, and a change, or difference in binding between the two
10 samples indicates the presence of an agent capable of binding to the cell cycle protein and potentially modulating its activity. That is, if the binding of the competitor is different in the second sample relative to the first sample, the agent is capable of binding to the cell cycle protein.

Alternatively, a preferred embodiment utilizes differential screening to identify drug candidates that bind to the native cell cycle protein, but cannot bind to modified cell cycle proteins. The structure of
15 the cell cycle protein may be modeled, and used in rational drug design to synthesize agents that interact with that site. Drug candidates that affect cell cycle bioactivity are also identified by screening drugs for the ability to either enhance or reduce the activity of the protein.

Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all
20 samples is for a time sufficient for the binding of the agent to the protein. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

A variety of other reagents may be included in the screening assays. These include reagents like
25 salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding.

30 Screening for agents that modulate the activity of cell cycle may also be done. In a preferred embodiment, methods for screening for a bioactive agent capable of modulating the activity of cell cycle comprise the steps of adding a candidate bioactive agent to a sample of a cell cycle protein (or cells comprising a cell cycle protein) and determining an alteration in the biological activity of the cell cycle protein. "Modulating the activity of a cell cycle protein" includes an increase in
35 activity, a decrease in activity, or a change in the type or kind of activity present. Thus, in this

embodiment, the candidate agent should both bind to cell cycle (although this may not be necessary), and alter its biological or biochemical activity as defined herein. The methods include both in vitro screening methods, as are generally outlined above, and in vivo screening of cells for alterations in the presence, distribution, activity or amount of cell cycle protein.

- 5 Thus, in this embodiment, the methods comprise combining an cell cycle sample and a candidate bioactive agent, and evaluating the effect on the cell cycle. By "cell cycle protein activity" or grammatical equivalents herein is meant one or more of the cell cycle protein's biological activities, including, but not limited to, its ability to affect the cell cycle, bind to a TRAF protein, phosphorylate proteins associated with microtubules and/or cell cycling, affect cell proliferation, mediate
10 apoptosis, mediate cellular activation, and/or modulate pathways involved in tumor growth.

In a preferred embodiment, the activity of the cell cycle protein is decreased; in another preferred embodiment, the activity of the cell cycle protein is increased. Thus, bioactive agents that are antagonists are preferred in some embodiments, and bioactive agents that are agonists may be preferred in other embodiments.

- 15 In a preferred embodiment, the invention provides methods for screening for bioactive agents capable of modulating the activity of an cell cycle protein. The methods comprise adding a candidate bioactive agent, as defined above, to a cell comprising cell cycle proteins. Preferred cell types include almost any cell. The cells contain a recombinant nucleic acid that encodes an cell cycle protein. In a preferred embodiment, a library of candidate agents are tested on a plurality of
20 cells.

- Detection of cell cycle regulation may be done as will be appreciated by those in the art. In one embodiment, indicators of the cell cycle are used. There are a number of parameters that may be evaluated or assayed to allow the detection of alterations in cell cycle regulation, including, but not limited to, cell viability assays, assays to determine whether cells are arrested at a particular cell
25 cycle stage ("cell proliferation assays"), and assays to determine at which cell stage the cells have arrested ("cell phase assays"). By assaying or measuring one or more of these parameters, it is possible to detect not only alterations in cell cycle regulation, but alterations of different steps of the cell cycle regulation pathway. This may be done to evaluate native cells, for example to quantify the aggressiveness of a tumor cell type, or to evaluate the effect of candidate drug agents
30 that are being tested for their effect on cell cycle regulation. In this manner, rapid, accurate screening of candidate agents may be performed to identify agents that modulate cell cycle regulation.

- Thus, the present compositions and methods are useful to elucidate bioactive agents that can cause a cell or a population of cells to either move out of one growth phase and into another, or
35 arrest in a growth phase. In some embodiments, the cells are arrested in a particular growth

phase, and it is desirable to either get them out of that phase or into a new phase. Alternatively, it may be desirable to force a cell to arrest in a phase, for example G1, rather than continue to move through the cell cycle. Similarly, it may be desirable in some circumstances to accelerate a non-arrested but slowly moving population of cells into either the next phase or just through the cell cycle, or to delay the onset of the next phase. For example, it may be possible to alter the activities of certain enzymes, for example kinases, phosphatases, proteases or ubiquitination enzymes, that contribute to initiating cell phase changes.

In a preferred embodiment, the methods outlined herein are done on cells that are not arrested in the G1 phase; that is, they are rapidly or uncontrollably growing and replicating, such as tumor cells. In this manner, candidate agents are evaluated to find agents that can alter the cell cycle regulation, i.e. cause the cells to arrest at cell cycle checkpoints, such as in G1 (although arresting in other phases such as S, G2 or M are also desirable). Alternatively, candidate agents are evaluated to find agents that can cause proliferation of a population of cells, i.e. that allow cells that are generally arrested in G1 to start proliferating again; for example, peripheral blood cells, terminally differentiated cells, stem cells in culture, etc.

Accordingly, the invention provides methods for screening for alterations in cell cycle regulation of a population of cells. By "alteration" or "modulation" (used herein interchangeably), is generally meant one of two things. In a preferred embodiment, the alteration results in a change in the cell cycle of a cell, i.e. a proliferating cell arrests in any one of the phases, or an arrested cell moves out of its arrested phase and starts the cell cycle, as compared to another cell or in the same cell under different conditions. Alternatively, the progress of a cell through any particular phase may be altered; that is, there may be an acceleration or delay in the length of time it takes for the cells to move through a particular growth phase. For example, the cell may be normally undergo a G1 phase of several hours; the addition of an agent may prolong the G1 phase.

The measurements can be determined wherein all of the conditions are the same for each measurement, or under various conditions, with or without bioactive agents, or at different stages of the cell cycle process. For example, a measurement of cell cycle regulation can be determined in a cell or cell population wherein a candidate bioactive agent is present and wherein the candidate bioactive agent is absent. In another example, the measurements of cell cycle regulation are determined wherein the condition or environment of the cell or populations of cells differ from one another. For example, the cells may be evaluated in the presence or absence or previous or subsequent exposure of physiological signals, for example hormones, antibodies, peptides, antigens, cytokines, growth factors, action potentials, pharmacological agents including chemotherapeutics, radiation, carcinogenics, or other cells (i.e. cell-cell contacts). In another example, the measurements of cell cycle regulation are determined at different stages of the cell cycle process. In yet another example, the measurements of cell cycle regulation are taken wherein the conditions are the same, and the alterations are between one cell or cell population

and another cell or cell population.

By a "population of cells" or "library of cells" herein is meant at least two cells, with at least about 10^3 being preferred, at least about 10^6 being particularly preferred, and at least about 10^8 to 10^9 being especially preferred. The population or sample can contain a mixture of different cell types from either primary or secondary cultures although samples containing only a single cell type are preferred, for example, the sample can be from a cell line, particularly tumor cell lines, as outlined below. The cells may be in any cell phase, either synchronously or not, including M, G1, S, and G2. In a preferred embodiment, cells that are replicating or proliferating are used; this may allow the use of retroviral vectors for the introduction of candidate bioactive agents. Alternatively, non-replicating cells may be used, and other vectors (such as adenovirus and lentivirus vectors) can be used. In addition, although not required, the cells are compatible with dyes and antibodies.

Preferred cell types for use in the invention include, but are not limited to, mammalian cells, including animal (rodents, including mice, rats, hamsters and gerbils), primates, and human cells, particularly including tumor cells of all types, including breast, skin, lung, cervix, colorectal, leukemia, brain, etc.

In a preferred embodiment, the methods comprise assaying one or more of several different cell parameters, including, but not limited to, cell viability, cell proliferation, and cell phase. Other parameters include TRAF protein binding, phosphorylation activity, particularly of those proteins associated with microtubules and/or cell cycling, apoptosis inducement or regulation, cellular activation, and/or involvement in pathways involved in tumor growth. In another embodiment, Mkinase may act to recruit a protein having phosphorylation (kinase) activity. For example, Mkinase may complex, associate or induce a protein which has kinase activity.

In yet another embodiment, Mkinase binds to any one of the following: Homo sapiens mRNA for CDC23- cell division cycle protein; RCC1- antioxidant protein 1(AOP1); Novel associated with ARF; Human helicase II (RAD54L); Apurinic/aprimidinic endonuclease (HAP); Novel with homology to ELK1 at DNA level; Homo sapiens lipoprotein receptor-related protein- AM2 receptor; LDL-receptor/low density lipoprotein-related protein; Homo sapiens putative G protein-coupled receptor; Human glycoprotein receptor gp330 precursor; A novel transmembrane protein in prostate cancer, TENB2; MOAT-D multidrug resistance-associate; evecin-1- contains PH domain; Homo sapiens nel- PKC binding protein; TPRD1- contains tetratricopeptide repeat; Homo sapiens GABA-B1a receptor; Homo sapiens mRNA for nuclear receptor co-repressor (hN-CoR); Ig-like protein- CD33L2; von Willebrand factor; Homo sapiens RIG-like 7-1 mRNA; Novel Zn finger protein associated with p27; Human menin (MEN1) gene- multiple endocrine neoplasia-type 1; KIAA1097- contains ubiquitin protease domain; Human family of notch2; Human Notch3; Human adenylate kinase 1 (hAK1); Human phosphate cytidylyltransferase; H.sapiens mRNA for epithelin/granulin; Human c-erb-B-2 mRNA; Homo sapiens WD40 protein Ciao1; Novel GTP-

binding protein; KIAA0618; KIAA0863; KIAA1064; and KIAA0275.

In a preferred embodiment, cell viability is assayed, to ensure that a lack of cellular change is due to experimental conditions (i.e. the introduction of a candidate bioactive agent) not cell death.

There are a variety of suitable cell viability assays which can be used, including, but not limited to,
5 light scattering, viability dye staining, and exclusion dye staining.

In a preferred embodiment, a light scattering assay is used as the viability assay, as is well known in the art. For example, when viewed in the FACS, cells have particular characteristics as measured by their forward and 90 degree (side) light scatter properties. These scatter properties represent the size, shape and granule content of the cells. These properties account for two
10 parameters to be measured as a readout for the viability. Briefly, the DNA of dying or dead cells generally condenses, which alters the 90° scatter; similarly, membrane blebbing can alter the forward scatter. Alterations in the intensity of light scattering, or the cell-refractive index indicate alterations in viability.

Thus, in general, for light scattering assays, a live cell population of a particular cell type is
15 evaluated to determine it's forward and side scattering properties. This sets a standard for scattering that can subsequently be used.

In a preferred embodiment, the viability assay utilizes a viability dye. There are a number of known viability dyes that stain dead or dying cells, but do not stain growing cells. For example, annexin V is a member of a protein family which displays specific binding to phospholipid (phosphatidylserine)
20 in a divalent ion dependent manner. This protein has been widely used for the measurement of apoptosis (programmed cell death) as cell surface exposure of phosphatidylserine is a hallmark early signal of this process. Suitable viability dyes include, but are not limited to, annexin, ethidium homodimer-1, DEAD Red, propidium iodide, SYTOX Green, etc., and others known in the art; see the Molecular Probes Handbook of Fluorescent Probes and Research Chemicals, Haugland, Sixth
25 Edition, hereby incorporated by reference; see Apoptosis Assay on page 285 in particular, and Chapter 16.

Protocols for viability dye staining for cell viability are known, see Molecular Probes catalog, supra. In this embodiment, the viability dye such as annexin is labeled, either directly or indirectly, and combined with a cell population. Annexin is commercially available, i.e., from PharMingen, San
30 Diego, California, or Caltag Laboratories, Millbrae, California. Preferably, the viability dye is provided in a solution wherein the dye is in a concentration of about 100 ng/ml to about 500 ng/ml, more preferably, about 500 ng/ml to about 1 µg/ml, and most preferably, from about 1 µg/ml to about 5 µg/ml. In a preferred embodiment, the viability dye is directly labeled; for example, annexin may be labeled with a fluorochrome such as fluorescein isothiocyanate (FITC), Alexa dyes, TRITC,
35 AMCA, APC, tri-color, Cy-5, and others known in the art or commercially available. In an alternate

preferred embodiment, the viability dye is labeled with a first label, such as a hapten such as biotin, and a secondary fluorescent label is used, such as fluorescent streptavidin. Other first and second labeling pairs can be used as will be appreciated by those in the art.

Once added, the viability dye is allowed to incubate with the cells for a period of time, and washed,
5 if necessary. The cells are then sorted as outlined below to remove the non-viable cells.

In a preferred embodiment, exclusion dye staining is used as the viability assay. Exclusion dyes are those which are excluded from living cells, i.e. they are not taken up passively (they do not permeate the cell membrane of a live cell). However, due to the permeability of dead or dying cells, they are taken up by dead cells. Generally, but not always, the exclusion dyes bind to DNA,
10 for example via intercalation. Preferably, the exclusion dye does not fluoresce, or fluoresces poorly, in the absence of DNA; this eliminates the need for a wash step. Alternatively, exclusion dyes that require the use of a secondary label may also be used. Preferred exclusion dyes include, but are not limited to, ethidium bromide; ethidium homodimer-1; propidium iodine; SYTOX green nucleic acid stain; Calcein AM, BCECF AM; fluorescein diacetate; TOTO® and TO-PRO™
15 (from Molecular Probes; supra, see chapter 16) and others known in the art.

Protocols for exclusion dye staining for cell viability are known, see the Molecular Probes catalog, supra. In general, the exclusion dye is added to the cells at a concentration of from about 100 ng/ml to about 500 ng/ml, more preferably, about 500 ng/ml to about 1 µg/ml, and most preferably, from about 0.1 µg/ml to about 5 µg/ml, with about 0.5 µg/ml being particularly preferred. The cells
20 and the exclusion dye are incubated for some period of time, washed, if necessary, and then the cells sorted as outlined below, to remove non-viable cells from the population.

In addition, there are other cell viability assays which may be run, including for example enzymatic assays, which can measure extracellular enzymatic activity of either live cells (i.e. secreted proteases, etc.), or dead cells (i.e. the presence of intracellular enzymes in the media; for example,
25 intracellular proteases, mitochondrial enzymes, etc.). See the Molecular Probes Handbook of Fluorescent Probes and Research Chemicals, Haugland, Sixth Edition, hereby incorporated by reference; see chapter 16 in particular.

In a preferred embodiment, at least one cell viability assay is run, with at least two different cell viability assays being preferred, when the fluors are compatible. When only 1 viability assay is run,
30 a preferred embodiment utilizes light scattering assays (both forward and side scattering). When two viability assays are run, preferred embodiments utilize light scattering and dye exclusion, with light scattering and viability dye staining also possible, and all three being done in some cases as well. Viability assays thus allow the separation of viable cells from non-viable or dying cells.

In addition to a cell viability assay, a preferred embodiment utilizes a cell proliferation assay. By

"proliferation assay" herein is meant an assay that allows the determination that a cell population is either proliferating, i.e. replicating, or not replicating.

In a preferred embodiment, the proliferation assay is a dye inclusion assay. A dye inclusion assay relies on dilution effects to distinguish between cell phases. Briefly, a dye (generally a fluorescent dye as outlined below) is introduced to cells and taken up by the cells. Once taken up, the dye is trapped in the cell, and does not diffuse out. As the cell population divides, the dye is proportionally diluted. That is, after the introduction of the inclusion dye, the cells are allowed to incubate for some period of time; cells that lose fluorescence over time are dividing, and the cells that remain fluorescent are arrested in a non-growth phase.

- 10 Generally, the introduction of the inclusion dye may be done in one of two ways. Either the dye cannot passively enter the cells (e.g. it is charged), and the cells must be treated to take up the dye; for example through the use of an electric pulse. Alternatively, the dye can passively enter the cells, but once taken up, it is modified such that it cannot diffuse out of the cells. For example, enzymatic modification of the inclusion dye may render it charged, and thus unable to diffuse out of the cells. For example, the Molecular Probes CellTracker™ dyes are fluorescent chloromethyl derivatives that freely diffuse into cells, and then glutathione S-transferase-mediated reaction produces membrane impermeant dyes.

- Suitable inclusion dyes include, but are not limited to, the Molecular Probes line of CellTracker™ dyes, including, but not limited to CellTracker™ Blue, CellTracker™ Yellow-Green, CellTracker™ Green, CellTracker™ Orange, PKH26 (Sigma), and others known in the art; see the Molecular Probes Handbook, supra; chapter 15 in particular.

- In general, inclusion dyes are provided to the cells at a concentration ranging from about 100 ng/ml to about 5 µg/ml, with from about 500 ng/ml to about 1 µg/ml being preferred. A wash step may or may not be used. In a preferred embodiment, a candidate bioactive agent is combined with the cells as described herein. The cells and the inclusion dye are incubated for some period of time, to allow cell division and thus dye dilution. The length of time will depend on the cell cycle time for the particular cells; in general, at least about 2 cell divisions are preferred, with at least about 3 being particularly preferred and at least about 4 being especially preferred. The cells are then sorted as outlined below, to create populations of cells that are replicating and those that are not. As will be appreciated by those in the art, in some cases, for example when screening for anti-proliferation agents, the bright (i.e. fluorescent) cells are collected; in other embodiments, for example for screening for proliferation agents, the low fluorescence cells are collected. Alterations are determined by measuring the fluorescence at either different time points or in different cell populations, and comparing the determinations to one another or to standards.

- 35 In a preferred embodiment, the proliferation assay is an antimetabolite assay. In general,

antimetabolite assays find the most use when agents that cause cellular arrest in G1 or G2 resting phase is desired. In an antimetabolite proliferation assay, the use of a toxic antimetabolite that will kill dividing cells will result in survival of only those cells that are not dividing. Suitable antimetabolites include, but are not limited to, standard chemotherapeutic agents such as methotrexate, cisplatin, taxol, hydroxyurea, nucleotide analogs such as AraC, etc. In addition, antimetabolite assays may include the use of genes that cause cell death upon expression.

The concentration at which the antimetabolite is added will depend on the toxicity of the particular antimetabolite, and will be determined as is known in the art. The antimetabolite is added and the cells are generally incubated for some period of time; again, the exact period of time will depend on the characteristics and identity of the antimetabolite as well as the cell cycle time of the particular cell population. Generally, a time sufficient for at least one cell division to occur.

In a preferred embodiment, at least one proliferation assay is run, with more than one being preferred. Thus, a proliferation assay results in a population of proliferating cells and a population of arrested cells. Moreover, other proliferation assays may be used, i.e., colorimetric assays known in the art.

In a preferred embodiment, either after or simultaneously with one or more of the proliferation assays outlined above, at least one cell phase assay is done. A "cell phase" assay determines at which cell phase the cells are arrested, M, G1, S, or G2.

In a preferred embodiment, the cell phase assay is a DNA binding dye assay. Briefly, a DNA binding dye is introduced to the cells, and taken up passively. Once inside the cell, the DNA binding dye binds to DNA, generally by intercalation, although in some cases, the dyes can be either major or minor groove binding compounds. The amount of dye is thus directly correlated to the amount of DNA in the cell, which varies by cell phase; G2 and M phase cells have twice the DNA content of G1 phase cells, and S phase cells have an intermediate amount, depending on at what point in S phase the cells are. Suitable DNA binding dyes are permeant, and include, but are not limited to, Hoechst 33342 and 33258, acridine orange, 7-AAD, LDS 751, DAPI, and SYTO 16, Molecular Probes Handbook, supra; chapters 8 and 16 in particular.

In general, the DNA binding dyes are added in concentrations ranging from about 1 µg/ml to about 5 µg/ml. The dyes are added to the cells and allowed to incubate for some period of time; the length of time will depend in part on the dye chosen. In one embodiment, measurements are taken immediately after addition of the dye. The cells are then sorted as outlined below, to create populations of cells that contain different amounts of dye, and thus different amounts of DNA; in this way, cells that are replicating are separated from those that are not. As will be appreciated by those in the art, in some cases, for example when screening for anti-proliferation agents, cells with the least fluorescence (and thus a single copy of the genome) can be separated from those that

are replicating and thus contain more than a single genome of DNA. Alterations are determined by measuring the fluorescence at either different time points or in different cell populations, and comparing the determinations to one another or to standards.

In a preferred embodiment, the cell phase assay is a cyclin destruction assay. In this embodiment, prior to screening (and generally prior to the introduction of a candidate bioactive agent, as outlined below), a fusion nucleic acid is introduced to the cells. The fusion nucleic acid comprises nucleic acid encoding a cyclin destruction box and a nucleic acid encoding a detectable molecule. "Cyclin destruction boxes" are known in the art and are sequences that cause destruction via the ubiquitination pathway of proteins containing the boxes during particular cell phases. That is, for example, G1 cyclins may be stable during G1 phase but degraded during S phase due to the presence of a G1 cyclin destruction box. Thus, by linking a cyclin destruction box to a detectable molecule, for example green fluorescent protein, the presence or absence of the detectable molecule can serve to identify the cell phase of the cell population. In a preferred embodiment, multiple boxes are used, preferably each with a different fluor, such that detection of the cell phase can occur.

A number of cyclin destruction boxes are known in the art, for example, cyclin A has a destruction box comprising the sequence RTVLGVIGD; the destruction box of cyclin B1 comprises the sequence RTALGDIGN. See Glotzer et al., *Nature* 349:132-138 (1991). Other destruction boxes are known as well: YMTVSIIDRFMQDSCVPKKMLQLVGVT (rat cyclin B); KFRLLQETMYMTVSIIDRFMQNSCVPKK (mouse cyclin B); RAILDWLIQVQMKFRLLQETMYMTVS (mouse cyclin B1); DRFLQAQLVCRKKLQVVGITALLASK (mouse cyclin B2); and MSVLRGKLQLVGTAAMLL (mouse cyclin A2).

The nucleic acid encoding the cyclin destruction box is operably linked to nucleic acid encoding a detectable molecule. The fusion proteins are constructed by methods known in the art. For example, the nucleic acids encoding the destruction box is ligated to a nucleic acid encoding a detectable molecule. By "detectable molecule" herein is meant a molecule that allows a cell or compound comprising the detectable molecule to be distinguished from one that does not contain it, i.e., an epitope, sometimes called an antigen TAG, a specific enzyme, or a fluorescent molecule. Preferred fluorescent molecules include but are not limited to green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), and enzymes including luciferase and β -galactosidase. When antigen TAGs are used, preferred embodiments utilize cell surface antigens. The epitope is preferably any detectable peptide which is not generally found on the cytoplasmic membrane, although in some instances, if the epitope is one normally found on the cells, increases may be detected, although this is generally not preferred. Similarly, enzymatic detectable molecules may also be used; for example, an enzyme that generates a novel or chromogenic product.

Accordingly, the results of sorting after cell phase assays generally result in at least two populations of cells that are in different cell phases.

The proteins and nucleic acids provided herein can also be used for screening purposes wherein the protein-protein interactions of the cell cycle proteins can be identified. Genetic systems have
5 been described to detect protein-protein interactions. The first work was done in yeast systems, namely the "yeast two-hybrid" system. The basic system requires a protein-protein interaction in order to turn on transcription of a reporter gene. Subsequent work was done in mammalian cells. See Fields et al., Nature 340:245 (1989); Vasavada et al., PNAS USA 88:10686 (1991); Fearon et al., PNAS USA 89:7958 (1992); Dang et al., Mol. Cell. Biol. 11:954 (1991); Chien et al., PNAS USA
10 88:9578 (1991); and U.S. Patent Nos. 5,283,173, 5,667,973, 5,468,614, 5,525,490, and 5,637,463. a preferred system is described in Serial Nos. 09/050,863, filed March 30, 1998 and 09/359,081 filed July 22, 1999, entitled "Mammalian Protein Interaction Cloning System". For use in conjunction with these systems, a particularly useful shuttle vector is described in Serial No. 09/133,944, filed August 14, 1998, entitled "Shuttle Vectors".

15 In general, two nucleic acids are transformed into a cell, where one is a "bait" such as the gene encoding a cell cycle protein or a portion thereof, and the other encodes a test candidate. Only if the two expression products bind to one another will an indicator, such as a fluorescent protein, be expressed. Expression of the indicator indicates when a test candidate binds to the cell cycle protein and can be identified as an cell cycle protein. Using the same system and the identified cell
20 cycle proteins the reverse can be performed. Namely, the cell cycle proteins provided herein can be used to identify new baits, or agents which interact with cell cycle proteins. Additionally, the two-hybrid system can be used wherein a test candidate is added in addition to the bait and the cell cycle protein encoding nucleic acids to determine agents which interfere with the bait, such as Traf4, and the cell cycle protein.

25 In one embodiment, a mammalian two-hybrid system is preferred. Mammalian systems provide post-translational modifications of proteins which may contribute significantly to their ability to interact. In addition, a mammalian two-hybrid system can be used in a wide variety of mammalian cell types to mimic the regulation, induction, processing, etc. of specific proteins within a particular
30 cell type. For example, proteins involved in a disease state (i.e., cancer, apoptosis related disorders) could be tested in the relevant disease cells. Similarly, for testing of random proteins, assaying them under the relevant cellular conditions will give the highest positive results. Furthermore, the mammalian cells can be tested under a variety of experimental conditions that may affect intracellular protein-protein interactions, such as in the presence of hormones, drugs,
35 growth factors and cytokines, radiation, chemotherapeutics, cellular and chemical stimuli, etc., that may contribute to conditions which can effect protein-protein interactions, particularly those involved in cancer.

Assays involving binding such as the two-hybrid system may take into account non-specific binding proteins (NSB).

Expression in various cell types, and assays for cell cycle activity are described above. The activity assays, such as having an effect on cell proliferation or microtubules, can be performed to
5 confirm the activity of cell cycle proteins which have already been identified by their sequence identity/similarity or binding to Traf4 as well as to further confirm the activity of lead compounds identified as modulators of Mkinase.

The components provided herein for the assays provided herein may also be combined to form
10 kits. The kits can be based on the use of the protein and/or the nucleic acid encoding the cell cycle proteins. In one embodiment, other components are provided in the kit. Such components include one or more of packaging, instructions, antibodies, and labels. Additional assays such as those used in diagnostics are further described below.

In this way, bioactive agents are identified. Compounds with pharmacological activity are able to
15 enhance or interfere with the activity of the cell cycle protein. The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host, as further described below.

The present discovery relating to the role of cell cycle proteins in the cell cycle thus provides methods for inducing or preventing cell proliferation in cells. In a preferred embodiment, the cell
20 cycle proteins, and particularly cell cycle protein fragments, are useful in the study or treatment of conditions which are mediated by the cell cycle proteins, i.e. to diagnose, treat or prevent cell cycle associated disorders. Thus, "cell cycle associated disorders" or "disease state" include conditions involving both insufficient or excessive cell proliferation including for example, cancer.

Thus, in one embodiment, cell cycle regulation in cells or organisms are provided. In one
25 embodiment, the methods comprise administering to a cell or individual in need thereof, a cell cycle protein in a therapeutic amount. Alternatively, an anti-cell cycle antibody that reduces or eliminates the biological activity of the endogenous cell cycle protein is administered. In another embodiment, a bioactive agent as identified by the methods provided herein is administered. Alternatively, the methods comprise administering to a cell or individual a recombinant nucleic acid
30 encoding an cell cycle protein. As will be appreciated by those in the art, this may be accomplished in any number of ways. In a preferred embodiment, the activity of cell cycle is increased by increasing the amount of cell cycle in the cell, for example by overexpressing the endogenous cell cycle or by administering a gene encoding a cell cycle protein, using known gene-therapy techniques, for example. In a preferred embodiment, the gene therapy techniques include the
35 incorporation of the exogenous gene using enhanced homologous recombination (EHR), for example as described in PCT/US93/03868, hereby incorporated by reference in its entirety.

Without being bound by theory, it appears that cell cycle protein is an important protein in the cell cycle. Accordingly, disorders based on mutant or variant cell cycle genes may be determined. In one embodiment, the invention provides methods for identifying cells containing variant cell cycle genes comprising determining all or part of the sequence of at least one endogeneous cell cycle genes in a cell. As will be appreciated by those in the art, this may be done using any number of sequencing techniques. In a preferred embodiment, the invention provides methods of identifying the cell cycle genotype of an individual comprising determining all or part of the sequence of at least one cell cycle gene of the individual. This is generally done in at least one tissue of the individual, and may include the evaluation of a number of tissues or different samples of the same tissue. The method may include comparing the sequence of the sequenced cell cycle gene to a known cell cycle gene, i.e. a wild-type gene.

The sequence of all or part of the cell cycle gene can then be compared to the sequence of a known cell cycle gene to determine if any differences exist. This can be done using any number of known sequence identity programs, such as Bestfit, etc. In a preferred embodiment, the presence of a difference in the sequence between the cell cycle gene of the patient and the known cell cycle gene is indicative of a disease state or a propensity for a disease state.

In one embodiment, the invention provides methods for diagnosing a cell cycle related condition in an individual. The methods comprise measuring the activity of cell cycle in a tissue from the individual or patient, which may include a measurement of the amount or specific activity of a cell cycle protein. This activity is compared to the activity of cell cycle from either a unaffected second individual or from an unaffected tissue from the first individual. When these activities are different, the first individual may be at risk for a cell cycle associated disorder. In this way, for example, monitoring of various disease conditions may be done, by monitoring the levels of the protein or the expression of mRNA therefor. Similarly, expression levels may correlate to the prognosis.

In one aspect, the expression levels of cell cycle protein genes are determined in different patient samples or cells for which either diagnosis or prognosis information is desired. Gene expression monitoring is done on genes encoding cell cycle proteins. In one aspect, the expression levels of cell cycle protein genes are determined for different cellular states, such as normal cells and cells undergoing apoptosis or transformation. By comparing cell cycle protein gene expression levels in cells in different states, information including both up- and down-regulation of cell cycle protein genes is obtained, which can be used in a number of ways. For example, the evaluation of a particular treatment regime may be evaluated: does a chemotherapeutic drug act to improve the long-term prognosis in a particular patient. Similarly, diagnosis may be done or confirmed by comparing patient samples. Furthermore, these gene expression levels allow screening of drug candidates with an eye to mimicking or altering a particular expression level. This may be done by making blochips comprising sets of important cell cycle protein genes, such as those of the present invention, which can then be used in these screens. These methods can also be done on the

protein basis; that is, protein expression levels of the cell cycle proteins can be evaluated for diagnostic purposes or to screen candidate agents. In addition, the cell cycle protein nucleic acid sequences can be administered for gene therapy purposes, including the administration of antisense nucleic acids, or the cell cycle proteins administered as therapeutic drugs.

- 5 Cell cycle protein sequences bound to biochips include both nucleic acid and amino acid sequences as defined above. In a preferred embodiment, nucleic acid probes to cell cycle protein nucleic acids (both the nucleic acid sequences having the sequences outlined in the Figures and/or the complements thereof) are made. The nucleic acid probes attached to the biochip are designed to be substantially complementary to the cell cycle protein nucleic acids, i.e. the target sequence
- 10 (either the target sequence of the sample or to other probe sequences, for example in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so
- 15 great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions, particularly high stringency conditions, as outlined herein.

- A "nucleic acid probe" is generally single stranded but can be partially single and partially double
- 20 stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. In general, the nucleic acid probes range from about 8 to about 100 bases long, with from about 10 to about 80 bases being preferred, and from about 30 to about 50 bases being particularly preferred. In some embodiments, much longer nucleic acids can be used, up to hundreds of bases (e.g., whole genes).

- 25 As will be appreciated by those in the art, nucleic acids can be attached or immobilized to a solid support in a wide variety of ways. By "immobilized" and grammatical equivalents herein is meant the association or binding between the nucleic acid probe and the solid support is sufficient to be stable under the conditions of binding, washing, analysis, and removal as outlined below. The binding can be covalent or non-covalent. By "non-covalent binding" and grammatical equivalents
- 30 herein is meant one or more of either electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment of a molecule, such as, streptavidin to the support and the non-covalent binding of the biotinylated probe to the streptavidin. By "covalent binding" and grammatical equivalents herein is meant that the two moieties, the solid support and the probe, are attached by at least one bond, including sigma bonds, pi bonds and coordination
- 35 bonds. Covalent bonds can be formed directly between the probe and the solid support or can be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the probe or both molecules. Immobilization may also involve a combination of covalent and non-

covalent interactions.

In general, the probes are attached to the biochip in a wide variety of ways, as will be appreciated by those in the art. As described herein, the nucleic acids can either be synthesized first, with subsequent attachment to the biochip, or can be directly synthesized on the biochip.

- 5 The biochip comprises a suitable solid substrate. By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of the nucleic acid probes and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates are very large, and include, but are not limited to, glass and modified or
- 10 functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, etc. In general, the substrates allow optical detection and do not appreciably show fluorescence.
- 15 In a preferred embodiment, the surface of the biochip and the probe may be derivatized with chemical functional groups for subsequent attachment of the two. Thus, for example, the biochip is derivatized with a chemical functional group including, but not limited to, amino groups, carboxy groups, oxo groups and thiol groups, with amino groups being particularly preferred. Using these functional groups, the probes can be attached using functional groups on the probes. For
- 20 example, nucleic acids containing amino groups can be attached to surfaces comprising amino groups, for example using linkers as are known in the art; for example, homo- or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). In addition, in some cases, additional linkers, such as alkyl groups (including substituted and heteroalkyl groups) may be used.
- 25 In this embodiment, oligonucleotides, corresponding to the nucleic acid probe, are synthesized as is known in the art, and then attached to the surface of the solid support. As will be appreciated by those skilled in the art, either the 5' or 3' terminus may be attached to the solid support, or attachment may be via an internal nucleoside.
- In an additional embodiment, the immobilization to the solid support may be very strong, yet non-
- 30 covalent. For example, biotinylated oligonucleotides can be made, which bind to surfaces covalently coated with streptavidin, resulting in attachment.

Alternatively, the oligonucleotides may be synthesized on the surface, as is known in the art. For example, photoactivation techniques utilizing photopolymerization compounds and techniques are used. In a preferred embodiment, the nucleic acids can be synthesized in situ, using well known

photolithographic techniques, such as those described in WO 95/25116; WO 95/35505; U.S. Patent Nos. 5,700,637 and 5,445,934; and references cited within, all of which are expressly incorporated by reference; these methods of attachment form the basis of the Affimetrix GeneChip™ technology.

- 5 "Differential expression," or grammatical equivalents as used herein, refers to both qualitative as well as quantitative differences in the genes' temporal and/or cellular expression patterns within and among the cells. Thus, a differentially expressed gene can qualitatively have its expression altered, including an activation or inactivation, in, for example, normal versus apoptotic cell. That is, genes may be turned on or turned off in a particular state, relative to another state. As is apparent
- 10 to the skilled artisan, any comparison of two or more states can be made. Such a qualitatively regulated gene will exhibit an expression pattern within a state or cell type which is detectable by standard techniques in one such state or cell type, but is not detectable in both. Alternatively, the determination is quantitative in that expression is increased or decreased; that is, the expression of the gene is either upregulated, resulting in an increased amount of transcript, or downregulated,
- 15 resulting in a decreased amount of transcript. The degree to which expression differs need only be large enough to quantify via standard characterization techniques as outlined below, such as by use of Affymetrix GeneChip™ expression arrays, Lockhart, Nature Biotechnology 14:1675-1680 (1996), hereby expressly incorporated by reference. Other techniques include, but are not limited to, quantitative reverse transcriptase PCR, Northern analysis and RNase protection.
- 20 As will be appreciated by those in the art, this may be done by evaluation at either the gene transcript, or the protein level; that is, the amount of gene expression may be monitored using nucleic acid probes to the DNA or RNA equivalent of the gene transcript, and the quantification of gene expression levels, or, alternatively, the final gene product itself (protein) can be monitored, for example through the use of antibodies to the cell cycle protein and standard immunoassays
- 25 (ELISAs, etc.) or other techniques, including mass spectroscopy assays, 2D gel electrophoresis assays, etc.

- In another method detection of the mRNA is performed *in situ*. In this method permeabilized cells or tissue samples are contacted with a detectably labeled nucleic acid probe for sufficient time to allow the probe to hybridize with the target mRNA. Following washing to remove the non-
- 30 specifically bound probe, the label is detected. For example a digoxigenin labeled riboprobe (RNA probe) that is complementary to the mRNA encoding an cell cycle protein is detected by binding the digoxigenin with an anti-digoxigenin secondary antibody and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

- In another preferred method, expression of cell cycle protein is performed using *in situ* imaging
- 35 techniques employing antibodies to cell cycle proteins. In this method cells are contacted with from one to many antibodies to the cell cycle protein(s). Following washing to remove non-specific

antibody binding, the presence of the antibody or antibodies is detected. In one embodiment the antibody is detected by incubating with a secondary antibody that contains a detectable label. In another method the primary antibody to the cell cycle protein(s) contains a detectable label. In another preferred embodiment each one of multiple primary antibodies contains a distinct and
5 detectable label. This method finds particular use in simultaneous screening for a plurality of cell cycle proteins. The label may be detected in a fluorometer which has the ability to detect and distinguish emissions of different wavelengths. In addition, a fluorescence activated cell sorter (FACS) can be used in this method. As will be appreciated by one of ordinary skill in the art, numerous other histological imaging techniques are useful in the invention and the antibodies can
10 be used in ELISA, immunoblotting (Western blotting), immunoprecipitation, BIACORE technology, and the like.

In one embodiment, the cell cycle proteins of the present invention may be used to generate polyclonal and monoclonal antibodies to cell cycle proteins, which are useful as described herein. Similarly, the cell cycle proteins can be coupled, using standard technology, to affinity
15 chromatography columns. These columns may then be used to purify cell cycle antibodies. In a preferred embodiment, the antibodies are generated to epitopes unique to the cell cycle protein; that is, the antibodies show little or no cross-reactivity to other proteins. These antibodies find use in a number of applications. For example, the cell cycle antibodies may be coupled to standard affinity chromatography columns and used to purify cell cycle proteins as further described below.
20 The antibodies may also be used as blocking polypeptides, as outlined above, since they will specifically bind to the cell cycle protein.

The anti-cell cycle protein antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an
25 adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the cell cycle protein or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin,
30 bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid a, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

The anti-cell cycle protein antibodies may, alternatively, be monoclonal antibodies. Monoclonal
35 antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or

are capable of producing antibodies that will specifically bind to the immunizing agent.

Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the cell cycle protein or a fusion protein thereof.

- Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin.
- 5
10
15
- Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the

- 20
- American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

- The culture medium in which the hybridoma cells are cultured can then be assayed for the
- 25
- presence of monoclonal antibodies directed against cell cycle protein. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard
- 30
- analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

- 35
- The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture

medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein a-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those
5 described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are
10 then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., *supra*] or by
15 covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are
20 well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

25 *In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

The anti-cell cycle protein antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric
30 immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or
35 rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues.

- Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or
- 5 substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].
- 10 Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986);
- 15 Riechmann et al., Nature, 332:323-327 (1988); Verhoeven et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are
- 20 typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

- Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the
- 25 preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)). Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely
- 30 resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10, 779-783 (1992); Lonberg et al., Nature 368 856-859 (1994); Morrison, Nature 368, 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996);
- 35 Neuberger, Nature Biotechnology 14, 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol. 13 65-93 (1995).

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the cell cycle protein, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

- 5 Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody
10 molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

- Antibody variable domains with the desired binding specificities (antibody-antigen combining sites)
15 can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted
20 into separate expression vectors, and are co-transfected into a suitable host organism. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

- Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate
25 antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [U.S. Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed
30 using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

- The anti-cell cycle protein antibodies of the invention have various utilities. For example, anti-cell
35 cycle protein antibodies may be used in diagnostic assays for an cell cycle protein, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and

- immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: a Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For
- 5 example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962);
- 10 David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

- Anti-Cell cycle protein antibodies also are useful for the affinity purification of cell cycle protein from recombinant cell culture or natural sources. In this process, the antibodies against cell cycle protein are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods
- 15 well known in the art. The immobilized antibody then is contacted with a sample containing the cell cycle protein to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the cell cycle protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the cell cycle protein from the antibody.

- 20 The anti-cell cycle protein antibodies may also be used in treatment. In one embodiment, the genes encoding the antibodies are provided, such that the antibodies bind to and modulate the cell cycle protein within the cell.

- In one embodiment, a therapeutically effective dose of an cell cycle protein, agonist or antagonist is administered to a patient. By "therapeutically effective dose" herein is meant a dose that produces
- 25 the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for cell cycle protein degradation, systemic versus localized delivery, as well as the age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation
- 30 by those skilled in the art.

A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals, and organisms. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

- 35 The administration of the cell cycle protein, agonist or antagonist of the present invention can be.

done in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly. In some instances, for example, in the treatment of wounds and inflammation, the composition may be directly applied as a solution or spray. Depending upon the manner of
5 introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

- The pharmaceutical compositions of the present invention comprise an cell cycle protein, agonist or antagonist (including antibodies and bioactive agents as described herein) in a form suitable for administration to a patient. In the preferred embodiment, the pharmaceutical compositions are in a
10 water soluble form, such as being present as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid,
15 propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. "Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum
20 salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine.
- 25 The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

- Combinations of the compositions may be administered. Moreover, the compositions may be
30 administered in combination with other therapeutics, including growth factors or chemotherapeutics and/or radiation. Targeting agents (i.e. ligands for receptors on cancer cells) may also be combined with the compositions provided herein.

- In one embodiment provided herein, the antibodies are used for immunotherapy, thus, methods of immunotherapy are provided. By "immunotherapy" is meant treatment of cell cycle protein related
35 disorders with an antibody raised against a cell cycle protein. As used herein, immunotherapy can be passive or active. Passive immunotherapy, as defined herein, is the passive transfer of

antibody to a recipient (patient). Active immunization is the induction of antibody and/or T-cell responses in a recipient (patient). Induction of an immune response can be the consequence of providing the recipient with an cell cycle protein antigen to which antibodies are raised. As appreciated by one of ordinary skill in the art, the cell cycle protein antigen may be provided by

5 injecting an cell cycle protein against which antibodies are desired to be raised into a recipient, or contacting the recipient with an cell cycle protein nucleic acid, capable of expressing the cell cycle protein antigen, under conditions for expression of the cell cycle protein antigen.

In a preferred embodiment, a therapeutic compound is conjugated to an antibody, preferably an cell cycle protein antibody. The therapeutic compound may be a cytotoxic agent. In this method,

10 targeting the cytotoxic agent to apoptotic cells or tumor tissue or cells, results in a reduction in the number of afflicted cells, thereby reducing symptoms associated with apoptosis, cancer cell cycle protein related disorders. Cytotoxic agents are numerous and varied and include, but are not limited to, cytotoxic drugs or toxins or active fragments of such toxins. Suitable toxins and their corresponding fragments include diphtheria A chain, exotoxin A chain, ricin A chain, abrin A chain,

15 curcin, crotin, phenomycin, enomycin and the like. Cytotoxic agents also include radiochemicals made by conjugating radioisotopes to antibodies raised against cell cycle proteins, or binding of a radionuclide to a chelating agent that has been covalently attached to the antibody.

In a preferred embodiment, cell cycle protein genes are administered as DNA vaccines, either single nucleic acids or combinations of cell cycle protein genes. Naked DNA vaccines are

20 generally known in the art; see Brower, Nature Biotechnology 16:1304-1305 (1998). Methods for the use of nucleic acids as DNA vaccines are well known to one of ordinary skill in the art, and include placing an cell cycle protein gene or portion of an cell cycle protein nucleic acid under the control of a promoter for expression in a patient. The cell cycle protein gene used for DNA vaccines can encode full-length cell cycle proteins, but more preferably encodes portions of the cell

25 cycle proteins including peptides derived from the cell cycle protein. In a preferred embodiment a patient is immunized with a DNA vaccine comprising a plurality of nucleotide sequences derived from a cell cycle protein gene. Similarly, it is possible to immunize a patient with a plurality of cell cycle protein genes or portions thereof, as defined herein. Without being bound by theory, following expression of the polypeptide encoded by the DNA vaccine, cytotoxic T-cells, helper T-

30 cells and antibodies are induced which recognize and destroy or eliminate cells expressing cell cycle proteins.

In a preferred embodiment, the DNA vaccines include a gene encoding an adjuvant molecule with the DNA vaccine. Such adjuvant molecules include cytokines that increase the immunogenic response to the cell cycle protein encoded by the DNA vaccine. Additional or alternative adjuvants

35 are known to those of ordinary skill in the art and find use in the invention.

For illustrative purposes, hela cells were transfected with flag tagged mkinase. An in vitro kinase

activity was performed wherein mkinase showed phosphorylation activity or phosphorylation recruiting or inducing activity. Moreover, anti-flag staining and DAPI localization assays were performed (data shown below).

- 5 It is understood that the invention can be varied. All references cited herein are expressly incorporated by reference in their entirety. Moreover, all sequences displayed, cited by reference or accession number in the references are incorporated by reference herein. The examples are for illustrative purposes only and are not meant to limit the invention.

EXAMPLES

EXAMPLE 1-EXPRESSION

- 10 Figure 3 shows the mRNA expression pattern of Mkinase wherein actin is used as a control. Mkinase is ubiquitously expressed and includes strong expression in the prostate, testis and ovaries where one would expect cell cycle activity/regulation to be occurring.

EXAMPLE 2-KINASE ACTIVITY

- 15 Figure 4 shows the results of an in vitro kinase assay wherein myelin basis protein (MBP) is used as the substrate. The assay was performed using Mkinase in an immuno complex assay by using a flag-tag. The results show that the complex has strong kinase activity indicating that Mkinase has kinase activity or or is an adaptor protein which recruits a kinase (or has "pseudo kinase activity). In a preferred embodiment herein, it is believed that Mkinase has kinase activity.

- 20 Figure 5 shows figures involving a full-length (FL) Mkinase and an N-terminal deleted (ND) Mkinase. Particularly, Figures 5A and 5B indicate the approximate kinase domain and nuclear localization signal (NLS) of Mkinase wherein 5A is FL and 5B is ND. 5C shows the results of an in vitro kinase activity using ND, FL or a control vector and 5D shows the results of a Western blot indicating the presence of ND and FL used in 5C. The Mkinase is in an immuno complex as discussed above.

25 EXAMPLE 3-LOCALIZATION

Figure 6 shows the localization of Mkinase in Hela cells wherein 6A and 6B show staining with an anti-flag and Figures 6C and 6D show staining with DAPI. The results show strong localization to the cytoplasm. Upon division, ubiquitous localization, most likely due to nuclear membrane breakdown.

30 EXAMPLE 4-ASSOCIATION

- Yeast two-hybrid screening was performed with Traf4 as a bait to identify Mkinase as a protein which interacts with Traf4. Mkinase was then used as a bait to identify the following peptides as peptides which bind to Mkinase: Homo sapiens mRNA for CDC23- cell division cycle protein; RCC1- antioxidant protein 1(AOP1); Novel associated with ARF; Human helicase II (RAD54L);
- 5 Apurinic/aprimidinic endonuclease (HAP); Novel with homology to ELK1 at DNA level; Homo sapiens lipoprotein receptor-related protein- AM2 receptor; LDL-receptor/low density lipoprotein-related protein; Homo sapiens putative G protein-coupled receptor; Human glycoprotein receptor gp330 precursor; A novel transmembrane protein in prostate cancer, TENB2; MOAT-D multidrug resistance-associate; evelin-1- contains PH domain; Homo sapiens nel- PKC binding protein;
- 10 TPRD1- contains tetratricopeptide repeat; Homo sapiens GABA-B1a receptor; Homo sapiens mRNA for nuclear receptor co-repressor (hN-CoR); Ig-like protein- CD33L2; von Willebrand factor; Homo sapiens RIG-like 7-1 mRNA; Novel Zn finger protein associated with p27; Human menin (MEN1) gene- multiple endocrine neoplasia-type 1; KIAA1097- contains ubiquitin protease domain; Human family of notch2; Human Notch3; Human adenylate kinase 1 (hAK1); Human
- 15 phosphate cytidylyltransferase; H.sapiens mRNA for epithelin/granulin; Human c-erb-B-2 mRNA; Homo sapiens WD40 protein Cioa1; Novel GTP-binding protein; KIAA0618; KIAA0863; KIAA1064; and KIAA0275.

EXAMPLE 5-CLONING

- Figures 1 and 2 show embodiments of the sequence of Mkinase. The start of Mkinase in Figure 2
- 20 is believed to begin at the first methionine as indicated in Figure 7. Figure 7A shows the approximate location of the domain in Mkinase that has homology to other known kinases. Figure 7B shows a multiple sequence alignment of Mkinase from amino acids 1 through 233 against corresponding regions of other known kinases.

CLAIMS

We claim:

1. A recombinant nucleic acid encoding a cell cycle protein comprising a nucleic acid that hybridizes under high stringency conditions to a sequence complementary to that set forth in
5 Figure 1.
2. The recombinant nucleic acid of claim 1 wherein said protein binds to Traf4.
3. The recombinant nucleic acid of claim 1 comprising a nucleic acid sequence as set forth in Figure 1.
4. A recombinant nucleic acid encoding a cell cycle protein comprising a nucleic acid having at
10 least 85% sequence identity to a sequence as set forth in Figure 1.
5. A recombinant nucleic acid encoding an amino acid sequence as shown in Figure 2.
6. An expression vector comprising the recombinant nucleic acid according to any one of claims 1, 2, 3, 4, or 5, operably linked to regulatory sequences recognized by a host cell transformed with the nucleic acid.
- 15 7. A host cell comprising the recombinant nucleic acid according to any one of claims 1, 2, 3, 4, or 5.
8. A host cell comprising the vector of claim 6.
9. A process for producing a cell cycle protein comprising culturing the host cell of claim 8 under conditions suitable for expression of a cell cycle protein.
- 20 10. A process according to claim 9 further comprising recovering said cell cycle protein.
11. A recombinant cell cycle protein encoded by the nucleic acid of any of claims 1, 2, 3, 4, or 5.
12. A recombinant polypeptide comprising an amino acid sequence having at least 80% sequence identity with the sequence set forth in Figure 2.
13. The recombinant polypeptide of claim 12 wherein said polypeptide binds to Traf4
- 25 14. The recombinant polypeptide of claim 12 wherein said sequence is set forth in Figure 2.

15. An isolated polypeptide which specifically binds to a cell cycle protein according to claim 13.
16. A polypeptide according to claim 15 that is an antibody.
17. A polypeptide according to claim 16 wherein said antibody is a monoclonal antibody.
18. The monoclonal antibody of claim 17 wherein said antibody reduces or eliminates the biological
5 function of said cell cycle protein.
19. A method for screening for a bioactive agent capable of binding to a cell cycle protein, said method comprising:
a) combining a cell cycle protein and a candidate bioactive agent; and
b) determining the binding of said candidate bioactive agent to said cell cycle protein.
- 10 20. A method for screening for a bioactive agent capable of interfering with the binding of a cell cycle protein and a Traf4 protein, said method comprising:
a) combining a cell cycle protein, a candidate bioactive agent and a Traf4 protein; and
b) determining the binding of said cell cycle protein and said Traf4 protein.
21. A method according to Claim 20, wherein said cell cycle protein and said Traf4 protein are
15 combined first.
22. A method for screening for a bioactive agent capable of modulating the activity of cell cycle protein, said method comprising:
a) adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding a cell cycle protein; and
20 b) determining the effect of said candidate bioactive agent on said cell.
23. A method according to Claim 22, wherein a library of candidate bioactive agents is added to a plurality of cells comprising a recombinant nucleic acid encoding a cell cycle protein.

1 CCCGGAGCTAAGGCGCCCGAACCCGCGGCGGCGGTGGGGACGATGTGGTTTTTTGCCCCG
61 GACCCGGTCCGGGACTTTCCGTTTCGAGCTCATCCCGAGCCCCCAGAGGGCGGCCTGCCC
121 GGGCCCTGGGCCCTGCACCGCGGCCGCAAGAAGGCCACAGGCAGCCCCGTGTCCATCTTC
181 GTCTATGATGTGAAGCCTGGCGCGGAARGAGCAGACCCAGGTGGCCAAAGCTGCCTTCAA
241 GCRGCTTCAAACTCTACGGCACCCCAACATCRCTGGCTTACATCGATGGACTGGAGACA
301 GAAAAATGCCTCCACGTCGTGACAGAGGCTGTGACCCCGTTGGGAATATACCTCAAGGCG
361 AGAGTGGAGGCTGGTGGCCTGAAGGAGCTGGAGATCTCCTGGGGGCTACACCAGATCGTG
421 AAAGCCCTCAGCTTCCTGGTCAACGACTGCAGCCTCATCCACAACAATGTCTGCATGGCC
481 GCCGTGTTCTGGACCGAGCTGGCGAGTGAAGCTTGGGGGCTGGACTACATGTATTCTG
541 GCCCAGGGCAACGGTGGGGGACCTCCCCGCAAGGGGATCCCCGAGCTTGAGCAGTATGAC
601 CCCCCGAGTTGGCTGACAGCAGTGGCAGAGTGGTCAGAGAGAAGTGGTCAGCAGACATG
661 TGGCGCTTGGGCTGCCTCATTTGGGAAGTCTTCAATGGGCCCCCTACCTCGGGCAGCAGCC
721 CTACGCAACCCTGGGAAGATCCCCAAAACGCTGGCGCCCCATTACTGTGAGCTGGTGGGA
781 GCAAACCCCAAGGTGCGTCCCAACCCAGCCCGCTTCTGCAGAACTGCCGGGCACCTGGT
841 GGCTTCATGAGCAACCGCTTTGTAGAAACCAACCTCTTCTGGAGGAGATTTCAGATCAAA
901 GAGCCAGCCGAGAAGCAAAAATTCTTCCAGGAGCTGAGCAAGAGCCTGGACGCATTCCCT
961 GAGGATTTCTGTGGCACAAGCTGTGCCCCAGCTGCTGACCGCCTTCGAGTTTCGGCAAT
1021 GCTGGGGCCGTTGTCTCACGCCCCCTTTCAAGTGGGCAAGTTCTCTGAGCGCTGAGGAG
1081 TATCAGCAGAAGATCATCCCTGTGGTGGTCAAGATGTTCTCATCCACTGACCGGGCCATG
1141 CGCATCCGCTCCTGCAGCAGATGGAGCAGTTCATCCAGTACCTTGACGAGCCAACAGTC
1201 AACACCCAGATCTTCCCCACGTCGTACATGGCTTCTTGACACCAACCCTGCCATCCGG
1261 GAGCAGACGGTCAAGTCCATGCTGCTCCTGGCCCCAAAGCTGAACGAGGCCAACCTCAAT
1321 GTGGAGCTGATGAAGCACTTTGCACGGCTACAGGCCAAGGATGAACAGGGCCCCATCCGC
1381 TGCAACACCACAGTCTGCCTGGGCAAAATCGGCTCCTACCTCAGTGCTAGCACCAGACAC
1441 AGGGTCCTTACCTCTGCCTTCAGCCGAGCCACTAGGGACCCGTTTGCACCGTCCCGGGTT
1501 GCGGGTGTCTGGGCTTTGCTGCCACCCACAACCTCTACTCAATGAACGACTGTGCCAG
1561 AAGATCCTGCCTGTGCTCTGCGGTCTCACTGTAGATCCTGAGAAATCCGTGCGAGACCAG
1621 GCCTTCAAGGCATTTTCGGAGCTTCTGTCCAAATTGGAGTCTGTGTCGGAGGACCCGACC
1681 CAGCTGGAGGAAGTGGAGAAGGATGTCCATGCAGCCTCCAGCCCTGGCATGGGAGGAGCC
1741 GCAGCTAGCTGGGCAGGCTGGGCCGTGACCGGGTCTCCTCACTCACCTCCAAGCTGATC
1801 CGTTCGCACCCCAACCACTGCCCCAACAGAAACCAACATTCCCCAAAGACCCACGCCTGAA
1861 GGAGTTCCTGCCCCAGCCCCCACCCTGTTCTGCCCCCTACAACCTCAGGCCACTGG
1921 GAGACGCAGGAGGAGGACAAGGACACAGCAGAGGACAGCAGCACTGCTGACAGATGGGAC

FIGURE 1A

1981 GACGAAGACTGGGGCAGCCTGGAGCAGGAGGCCGAGTCTGTGCTGGCCCAGCAGGACGAC
2041 TGGAGCACCGGGGGCCAAGTGAGCCGTGCTAGTCAGGTCAGCAACTCCGACCACAAATCC
2101 TCCAAATCCCCAGAGTCCGACTGGAGCAGCTGGGAAGCTGAGGGCTCCTGGGAACAGGGC
2161 TGGCAGGAGCCAAGCTCCCAGGAGCCACCTTCTGACGGTACACGGCTGGCCAGCGAGTAT
2221 AACTGGGGTGGCCCAGAGTCCAGCGACAAGGGCGACCCCTTCGCTACCTGTCTGCACGT
2281 CCCAGCACCCAGCCGAGGCCAGACTCTTGGGGTGAGGACAAC TGGGAGGGCCTCGAGACT
2341 GACAGTCGACAGGTCAAGGCTGAGCTGGCCCCGGAAGAAGCGCGAGGAGCGGCGGGGAG
2401 ATGGAGGCCAAACGCGCCGAGAGGAAGGTGGCCAAGGGCCCCATGAAGCTGGGAGCCCCG
2461 AAGCTGGACTGAACCGTGGCGGTGGCCCTTCCCGGTGCGGAGAGCCCGCCCCACAGATG
2521 TATTTATTGTACAAACCATGTGAGCCCGGCCGCCAGCCAGGCCATCTCACGTGTACAT
2581 AATCAGAGCCACAATAAATTCTATTTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACCT
2641 CGAG

FIGURE 1B

1 NSGNNAEEAPGAKAPEPAAAVGTMWFFARDPVRDFPFELIPEPPEGGLPGPWALHRGRKK
61 ATGSPVSIFVYDVKPGAEETQVAKAAFKRFKTLRHPNILAYIDGLETEKCLHVVEAVT
121 PLGIYLKARVEAGGLKELEISWGLHQIVKALSFLVNDCSLIHNNVCMMAAVFVDRAGEWKL
181 GGLDYMYS AQNGGGPPRKGIPELEQYDPP ELADSSGRVVREKWSADMWRLGCLIWEVFN
241 GPLPRAAALRNPGKIPKTLAPHYCELVGANPKVRPNPARFLQNCRAPGGFMSNRFVETNL
301 FLEEQIKEPAEKQKFFQELSKSLDAPPEDFCRHKLLPQLLTAFEFGNAGAVVLTPLFKV
361 GKFLSAEEYQKKIIPVVVKMFSSTDRAMRIRLLQQMEQFIQYLDEPTVNTQIFPHVVHGF
421 LDTNPAIREQTVKSMLLLAPKLNANLNVELMKHFARLQAKDEQGPIRCNTTVCLGKIGS
481 YLSASTRHRVLTSAFSRATRDPFAPSRVAGVLGFAATHNLYSMNDCAQKILPVLGCLTVD
541 PEKSVRDQAFKAFRSFLSKLESVSEDPTQLEEEVKDVHAASSPGMGGAASWAGWAVTGV
601 SSLTSKLIRSHPTTAPTETNIPQRPTPEGVPAPAPTPVPATPTTSGHWETQEEDKDTAED
661 SSTADRWDDEDWGSLEQEAESVLAQQDDWSTGGQVSRASQVSNSDHKSSKSPESDWSSWE
721 AEGSWEQGWQEPSSQEPPSDGTRLASEYNWGGPESDKGDPFATLSARPSTQPRPDSWGE
781 DNWEGLETDSRQVKAELARKKREERRREMEAKRAERKVAKGPMKLGARKLDZ

FIGURE 2

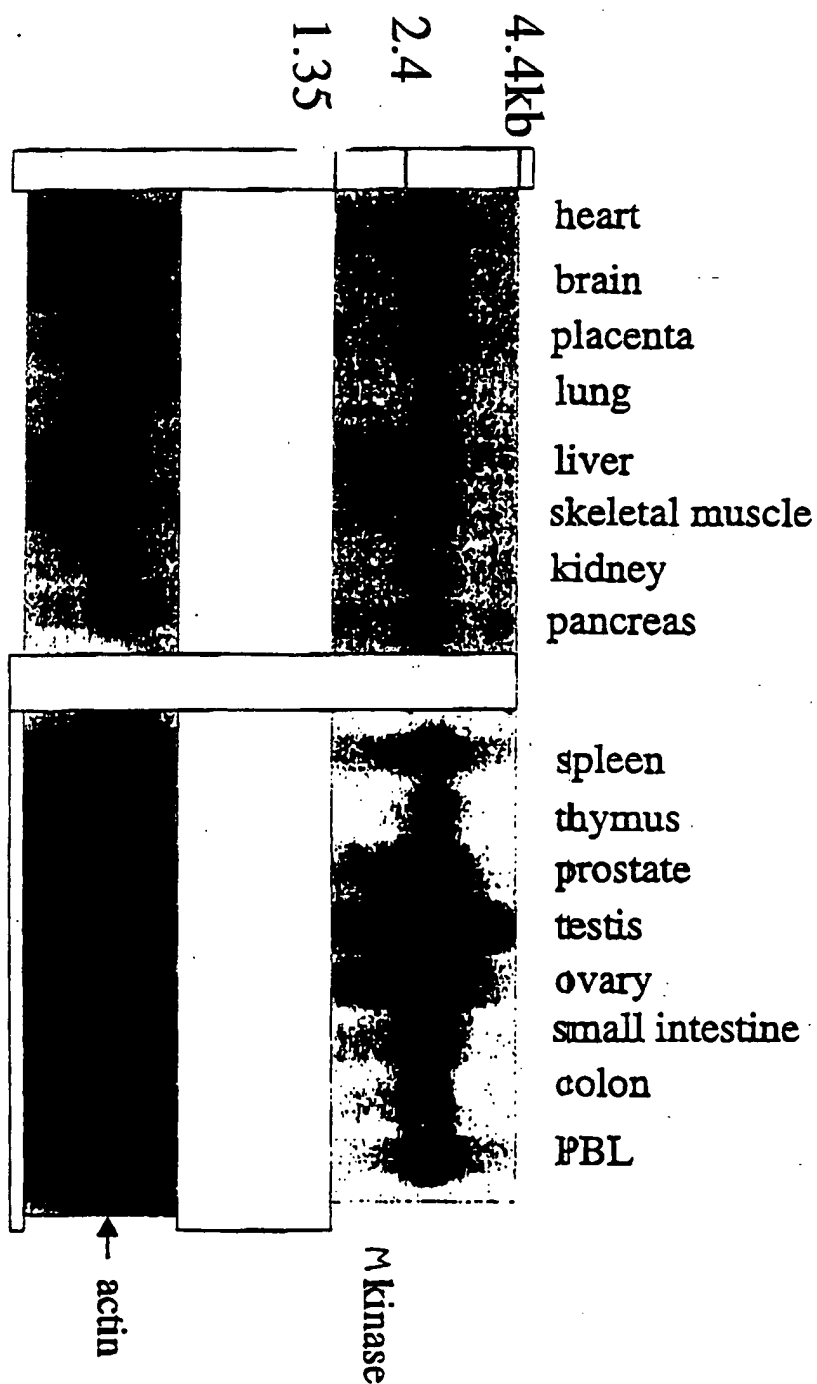
**FIGURE 3**

FIGURE 4

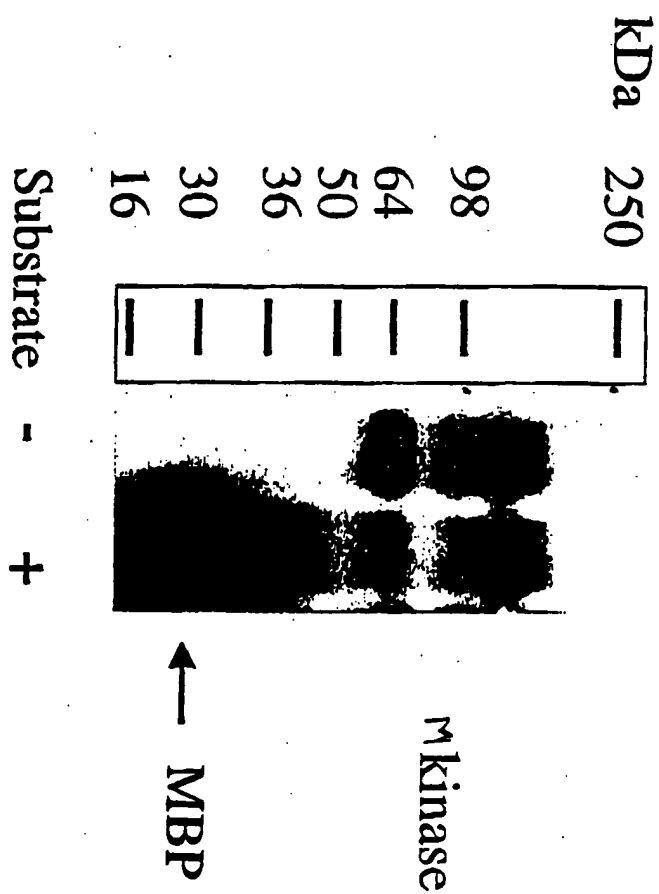


FIGURE 5A

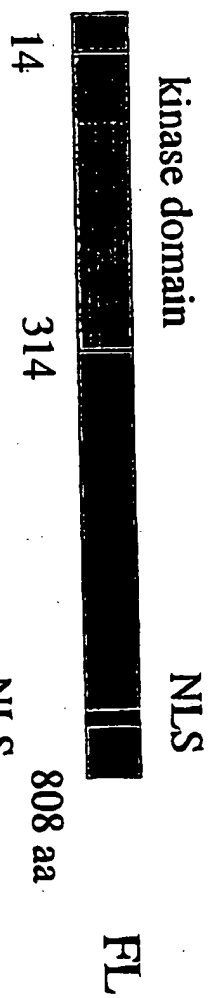
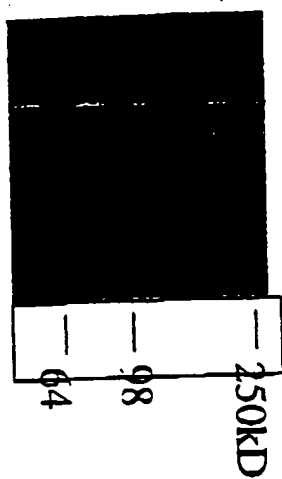


FIGURE 5B



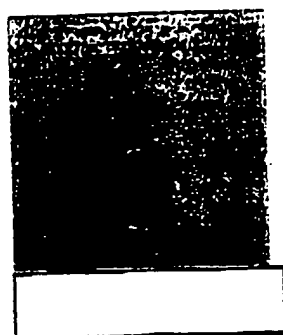
ND FL Vect.

FIGURE 5C



ND FL

FIGURE 5D



Anti-Flag staining

FIGURE 6A

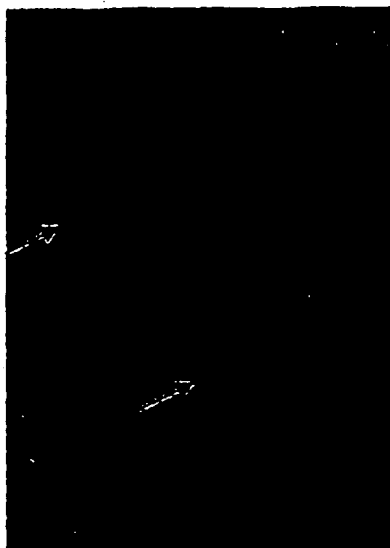


FIGURE 6B



DAPI

FIGURE 6C



FIGURE 6D



Kinase "Homologue" Domain

808aa

FIGURE 7A

FIGURE 7B

	Phosphate	Anchor
<p> AAA83272 AAA967437 AA72103/MAPK G01025 P51954 h.kinase </p>	<p> MSRSBARNDQVHVGKTLTKTKGNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 163 MSRSBARNDQVHVGKTLTKTKGNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 211 RCNSISATDEQPHIGVNLQRTIGKNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 101 INGRNSATSDQPHIGVNLQRTIGKNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 61 -----AKTVLQRTIGKSPG-----KVLAKVITQNEV-AIKIIDTALNPSEL 45 ---KMTFADQVHVGKTLTKTKGNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 58 GPSV-----HKR39 </p>	<p> MSRSBARNDQVHVGKTLTKTKGNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 163 MSRSBARNDQVHVGKTLTKTKGNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 211 RCNSISATDEQPHIGVNLQRTIGKNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 101 INGRNSATSDQPHIGVNLQRTIGKNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 61 -----AKTVLQRTIGKSPG-----KVLAKVITQNEV-AIKIIDTALNPSEL 45 ---KMTFADQVHVGKTLTKTKGNFA-----KVLAKVITQNEV-AIKIIDTALNPSEL 58 GPSV-----HKR39 </p>
<p> AAA83272 AAA967437 AA72103/MAPK G01025 P51954 h.kinase </p>	<p> OKLFRVETIKKQLODHPNIVLVYGVSTEGTCLVLETAAGQAVFDITLVAR-GNRKKEA 221 OKLFRVETIKKQLODHPNIVLVYGVSTEGTCLVLETAAGQAVFDITLVAR-GNRKKEA 269 OKLFRVETIKKQLODHPNIVLVYGVSTEGTCLVLETAAGQAVFDITLVAR-GNRKKEA 159 OKLFRVETIKKQLODHPNIVLVYGVSTEGTCLVLETAAGQAVFDITLVAR-GNRKKEA 119 QESNRVAVLANHSHRNIVGYKSFEBNGSLYVNDYCGGDLFNINQKCALPQEDQI 105 GVAKALVGRVATLHNPILAYIDGLTERKCLAVTEVATPLOTTLARVZAGOLELEIS 118 </p>	<p> OKLFRVETIKKQLODHPNIVLVYGVSTEGTCLVLETAAGQAVFDITLVAR-GNRKKEA 221 OKLFRVETIKKQLODHPNIVLVYGVSTEGTCLVLETAAGQAVFDITLVAR-GNRKKEA 269 OKLFRVETIKKQLODHPNIVLVYGVSTEGTCLVLETAAGQAVFDITLVAR-GNRKKEA 159 OKLFRVETIKKQLODHPNIVLVYGVSTEGTCLVLETAAGQAVFDITLVAR-GNRKKEA 119 QESNRVAVLANHSHRNIVGYKSFEBNGSLYVNDYCGGDLFNINQKCALPQEDQI 105 GVAKALVGRVATLHNPILAYIDGLTERKCLAVTEVATPLOTTLARVZAGOLELEIS 118 </p>
<p> AAA83272 AAA967437 AA72103/MAPK G01025 P51954 h.kinase </p>	<p> RAEPRQIVSAVQ-YLMSHUIIHRDLKACILLDQDMIRIKIDFG-----FANTFSLGNRLDT 277 RAEPRQIVSAVQ-YLMSHUIIHRDLKACILLDQDMIRIKIDFG-----FANTFSLGNRLDT 325 RAEPRQIVSAVQ-YLMSHUIIHRDLKACILLDQDMIRIKIDFG-----FANTFSLGNRLDT 215 RAEPRQIVSAVQ-YLMSHUIIHRDLKACILLDQDMIRIKIDFG-----FANTFSLGNRLDT 175 LDMPVQICLALK-HVHDEKILHRDLSQHTF7KQGVQGDG-----IARVLSFVETLAR 161 WGLK-QIVTALSPFLVN-----DCSLIINNVCHNAVVDSDHKLQGLD--YNYGAGONG 170 DRAQ-----MAK149 </p>	<p> RAEPRQIVSAVQ-YLMSHUIIHRDLKACILLDQDMIRIKIDFG-----FANTFSLGNRLDT 277 RAEPRQIVSAVQ-YLMSHUIIHRDLKACILLDQDMIRIKIDFG-----FANTFSLGNRLDT 325 RAEPRQIVSAVQ-YLMSHUIIHRDLKACILLDQDMIRIKIDFG-----FANTFSLGNRLDT 215 RAEPRQIVSAVQ-YLMSHUIIHRDLKACILLDQDMIRIKIDFG-----FANTFSLGNRLDT 175 LDMPVQICLALK-HVHDEKILHRDLSQHTF7KQGVQGDG-----IARVLSFVETLAR 161 WGLK-QIVTALSPFLVN-----DCSLIINNVCHNAVVDSDHKLQGLD--YNYGAGONG 170 DRAQ-----MAK149 </p>
<p> AAA83272 AAA967437 AA72103/MAPK G01025 P51954 h.kinase </p>	<p> PC-GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 333 PC-GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 381 PC-GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 271 PC-GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 231 TCIGTPTYL9-PEICENK-----PYNNKSDVNLQCVLVECTLGAFAQGNQDLVLTIS 217 ---GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 233 CTLI--MAK208 </p>	<p> PC-GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 333 PC-GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 381 PC-GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 271 PC-GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 231 TCIGTPTYL9-PEICENK-----PYNNKSDVNLQCVLVECTLGAFAQGNQDLVLTIS 217 ---GSPRYAA-PZLFGQKRYDQEV-----DVMSLGVLTLYTVSGSLPFDQGNLKLRENVLA 233 CTLI--MAK208 </p>

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/40987

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/47 C07K16/18 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE, BIOTECHNOLOGY ABS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 05175 A (MATOSSIAN ROGERS ARPI) 4 February 1999 (1999-02-04) abstract; claims 1-29; figure 7	1,6-11, 19
X	US 5 645 999 A (KOFF ANDREW C ET AL) 8 July 1997 (1997-07-08) abstract; claims 1-8	19,22,23
X	WO 97 07134 A (GENENTECH INC ;TULARIK INC (US)) 27 February 1997 (1997-02-27) abstract; claims 1-38; figure 2	19,22,23
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *A* document member of the same patent family

Date of the actual completion of the international search

14 February 2001

Date of mailing of the international search report

27/02/2001

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/40987

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KRAJEWSKA MARYLA ET AL: "TRAF-4 expression in epithelial progenitor cells. Analysis in normal adult, fetal, and tumor tissues." AMERICAN JOURNAL OF PATHOLOGY, vol. 152, no. 6, June 1998 (1998-06), pages 1549-1561, XP000982100 ISSN: 0002-9440 the whole document	20,21
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Y	--- REGNIER CATHERINE H ET AL: "Presence of a New Conserved Domain in CART1, a Novel Member of the Tumor Necrosis Factor Receptor-associated Protein Family, Which Is Expressed in Breast Carcinoma." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 43, 1995, pages 25715-25721, XP002160268 ISSN: 0021-9258 abstract	20,21
A	--- LETWIN K ET AL: "A MAMMALIAN DUAL SPECIFICITY PROTEIN KINASE NEK1 IS RELATED TO THE NIMA CELL CYCLE REGULATOR AND HIGHLY EXPRESSED IN MEIOTIC GERM CELLS" EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 11, no. 10, 1992, pages 3521-3531, XP002160269 ISSN: 0261-4189 cited in the application abstract; figures 1,2	1-14
P,X	--- WO 00 26670 A (BOEHRINGER INGELHEIM PHARMA) 11 May 2000 (2000-05-11) the whole document	19-23

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 15

Claim 15 relating to binding polypeptides to the polypeptide of claim 15 could not be searched as its subject-matter was insufficiently disclosed

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/40987

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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International Bureau



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C07K 14/47, 16/18, G01N 33/68

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(74) Agents: BREZNER, David, J. et al.; Flehr Hohbach Test Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

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(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US Not furnished (CIP)
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Published:
— with international search report
— with amended claims

(71) Applicant (for all designated States except US): RIGEL PHARMACEUTICALS, INC. [US/US]; 240 East Grand Avenue, South San Francisco, CA 94080 (US).

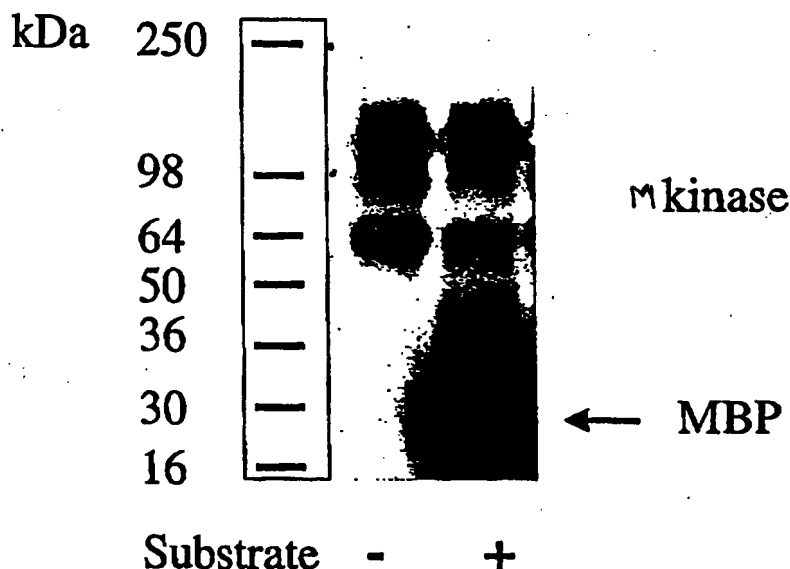
Date of publication of the amended claims: 11 October 2001

(72) Inventors; and

(75) Inventors/Applicants (for US only): LUO, Ying

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: TRAF4 ASSOCIATED CELL CYCLE PROTEINS, COMPOSITIONS AND METHODS OF USE



(57) Abstract: The present invention is directed to novel polypeptides, nucleic acids and related molecules which have an effect on or are related to the cell cycle. Also provided herein are vectors and host cells comprising those nucleic acid sequences, chimeric polypeptide molecules comprising the polypeptides of the present invention fused to heterologous polypeptide sequences, antibodies which bind to the polypeptides of the present invention and to methods for producing the polypeptides of the present invention. Further provided by the present invention are methods for identifying novel compositions which mediate cell cycle bioactivity, and the use of such compositions in diagnosis and treatment of disease.

WO 01/21799 A1

AMENDED CLAIMS

[received by the International Bureau on 27 April 2001 (27.04.01);
original claims 19-22 amended; remaining claims unchanged (1 page)]

15. An isolated polypeptide which specifically binds to a cell cycle protein according to claim 13.
16. A polypeptide according to claim 15 that is an antibody.
17. A polypeptide according to claim 16 wherein said antibody is a monoclonal antibody.
- 5 18. The monoclonal antibody of claim 17 wherein said antibody reduces or eliminates the biological function of said cell cycle protein.
- 10 19. A method for screening for a bioactive agent capable of binding to a cell cycle protein, said method comprising:
a) combining a cell cycle protein and a candidate bioactive agent; and
b) determining the binding of said candidate bioactive agent to said cell cycle protein;
wherein said cell cycle protein is encoded by a recombinant nucleic acid according to Claim 4.
- 15 20. A method for screening for a bioactive agent capable of interfering with the binding of a cell cycle protein and a Traf4 protein, said method comprising:
a) combining a cell cycle protein, a candidate bioactive agent and a Traf4 protein; and
b) determining the binding of said cell cycle protein and said Traf4 protein;
wherein said cell cycle protein is encoded by a recombinant nucleic acid according to Claim 4.
- 20 21. A method according to Claim 20, wherein said cell cycle protein and said Traf4 protein are combined first.
- 25 22. A method for screening for a bioactive agent capable of modulating the activity of cell cycle protein, said method comprising:
a) adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid according to Claim 4; and
b) determining the effect of said candidate bioactive agent on said cell.
- 30 23. A method according to Claim 22, wherein a library of candidate bioactive agents is added to a plurality of cells comprising a recombinant nucleic acid according to Claim 4.